BENCH TO HEALTH: INTEGRATION OF 3D ENTEROIDS TO MONITOR INFECTIOUS NOROVIRUS AND INFORM MICROBIAL RISK ASSESSMENT

by

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A dissertation submitted to Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore, Maryland

March 2021

ABSTRACT

Human noroviruses (HuNoVs) are the leading cause of acute gastroenteritis globally and cause approximately 200,000 deaths and $64 billion in economic losses each year. HuNoVs are highly transmissible and pose unique challenges for research and detection. The most significant of these challenges is the historical lack of a reproducible cell culture system for propagating infectious HuNoVs. The recent development of HuNoV cultivation in human intestinal enteroids (HIEs) now enables detection of infectious HuNoV. However, this HIE approach has not been adapted for use in monitoring HuNoV in the environment. Identification of infectious HuNoVs from the environment can increase monitoring accuracy, enhance risk estimates, and help prevent outbreaks. The goal of this dissertation was to lay the groundwork for use of the HuNoV HIE cell culture method in environmental monitoring scenarios and ultimately to advance quantitative microbial risk assessments (QMRAs). My research documented that HIEs can be used to cultivate HuNoV recovered from fomites relevant to HuNoV transmission in health care settings. A QMRA was then developed to model the risk of HuNoV illness for environmental service workers (ESWs) in healthcare settings caused by exposure from a single fomite contact in rooms with a HuNoV positive patient. ESWs face significant occupational health risks during HuNoV outbreaks and may also play an important role in transmitting the virus. The QMRA revealed that diarrheal events may drive fomite transmission of HuNoV. In sum, this dissertation provides a blueprint for using the HIE system to grow infectious HuNoVs recovered from the environment and presents a novel QMRA model that can be adapted and integrated with future measures to isolate and identify infectious HuNoV. This work fills important gaps in HuNoV

monitoring and risk assessment and provides approaches and tools to improve HuNoV detection, lead to more accurate predictions of health outcomes, and better evaluate control measures designed to reduce the HuNoV burden on human health.

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DEDICATION

To my village –

"No one is a failure who has friends"

To the researchers, clinicians, support staff, and all others who gave their time, energy, and lives to help the world through COVID-19, I offer words inspired by my advisor, Kellogg Schwab –

May your negative controls be negative,

may your positive controls be positive,

may you stay strong, and never give up.

ACKNOWLEDGEMENTS

First, I want to thank my astounding advisor, Kellogg Schwab. I don't think that I could have designed a more perfect person to serve as my mentor through the insanity that is a PhD, let alone during a global pandemic. From day one Kellogg has been a source of guidance, inspiration, and support and has taught me how to be a better scientist, leader, and person. Kellogg's meticulous approach to science (including an impeccable file naming system) and his dedication to helping people allowed me to not only complete a dissertation, but to thrive through the process. I will take many lessons that I have learned from Kellogg with me for the rest of my life, the most important of which will always be "never give up, and don't forget to wash your hands!"

I would also like to acknowledge my fabulous advisory committee – Nicholas Zachos, Keeve Nachman, Joseph Jacangelo, and Ghassan Hamra. Their support and guidance from early on helped shaped this dissertation in to a comprehensive work that I am proud to put out in the world. Nick – thank you for your endless help with enteroid work, it truly wouldn't have been possible without you. Your willingness to answer questions and troubleshoot problems taught me so much and I was able to grow norovirus thanks to your help. Keeve – your thoughtful advice on risk assessment allowed me to learn a brand-new skill and gave me the confidence to keep at it, even when it felt like all I was writing were

assumptions. Joe – your thoughtful feedback is evident throughout this work and your ability to always bring us back to real world applications is a lesson I will hold with me well beyond my time at Hopkins. Ghassan – thank you for jumping in to my committee headfirst and for being willing to answer my panicked statistics emails with thorough and informative responses.

In addition to Kellogg and my committee, there are a multitude of staff and faculty at Johns Hopkins who helped me on my path to getting a doctorate. First, I have to thank Katie Phipps who has been an amazing support system for the students in our department. I am lucky to call Katie a friend and am so grateful for the support, laughter, and Finn snuggles that she has brought in to my life. I also want to thank Roni Neff for taking a chance on this microbiologist and giving me an opportunity to pursue work completely different than my dissertation. My time working with Roni was an absolute joy and I am infinitely grateful for the skills I learned under her guidance. I want to acknowledge Natalie Exum for being such a kind mentor and someone I look up to as a scientist and person. Natalie has a true gift for science and an uncanny ability to bring out the best in those around her. I also want to thank Patty Poole who was an anchor in the department for my first few years and saved me many a time with her patience and know-how. Additionally, I am grateful for all of the staff, students, and faculty in the EHE department – it has been a joy learning from you all these past five years.

Caroline Coulter offered endless laboratory support and friendship through my time at Hopkins and the work in this dissertation happened because of her willingness to help out and go above and beyond. Caroline – you have saved my butt so many times and you made the burden of tending to my enteroid children a bit easier.

I would also like to acknowledge those faculty who served on my oral qualifying exam committees in addition to Kellogg and my committee – Ana Rule, Meghan Davis, Ed Bouwer, Bruce Lee, and Mary Fox. Ana is always a joy to see around campus and I am grateful for her welcoming presence. Meghan was a source of guidance and made it clear she was always ready to support students as they navigate Hopkins, their careers, and life. The late Ed Bouwer was truly a delight and I learned so much even in the limited interactions we had. I will never again draw containers without top closures on them or arrows that don't connect to their destination, especially when engineers are around.

Through my doctoral work I took up many hobbies that helped me relax, learn new things, and just take a break. One of the most significant of these diversions was improv comedy. For that, I have to thank Michael Hartwell, whose ability to take a bunch of scientists and turn them into an improv troupe is beyond astounding. I fell completely in love with everything improv has to offer and

Michael was a key driver in my love for the art. I also want to thank all of the fabulous improvisers I have met – Kristen, Cory, Ryan, Cassiel, Dan, Susan, Chuck, Brian, the members of Bacteria House Party, Silly Stories, Vanilla Bean, Game Knights, and all of the countless improvisers I have had the joy of meeting in just a few short years – thank you for having my back!

Another hobby that became a bit of an obsession was crochet, and for that I have to give Ashley Newton a special acknowledgement. The number of items I have crocheted in my time at Hopkins is probably enough work to justify a second doctorate and none of that would have been possible without Ashley's willingness to teach me. Thank you, Ashley, for being a patient teacher, a supportive desk neighbor, and a wonderful craft buddy. In that vein, I have to acknowledge the many members of the Lady Scholars club who were a constant source of joy, laughter, and support through this work. Alex, you helped get me through the day-to-day grind, offered support and vegan snacks when I was sad, and you were always willing to wear too much glitter and dance your butt off with me. Dana, I look up to you as a scientist and I will always cherish our antics and your "blurry graphs." Emily Illingworth, you were the best VP a girl could ask for and I remain constantly inspired by your tenacity and penchant to bring joy to those around you. Tara, I admire your skills as a scientist and your willingness to push back against problems and stand up for what you believe in. Emily Cooksey, I am so lucky we met and even luckier that we keep in touch – chatting

with you is always a delight and you have helped me feel less alone in all of this. Brooke – getting to know you has been an absolute pleasure and you win the award for best work accountability buddy.

I also have so many friends and family who have offered guidance and support and helped me become the person (and Doctor!) that I am today. Sass, Aaron, and Shannon – you all are family to me and it is a privilege to grow with you. Kaitlyn – there aren't really words to describe how lucky I feel to have been your roommate. You were and are the most perfect house goblin and both quarantine and the before times would have been a lot drearier without you. Katherine – you were my first friend in Baltimore and there is no one else I would have rather learned this city with. Cory – you're a killer podcast host and an even better friend, thank you for your constant support and delightful wit.

To mom, what do I even say? You have been there every step of my 24-year schooling journey and I cannot even begin to thank you for all you have done for me. Thank you for teaching me to be strong, to trust myself, and, if all else fails, to look up at the moon and calm the hell down.

To dad, you fostered my curiosity and love of science from a young age and I wouldn't have made it this far without you. I am forever grateful for your goofy

sense of humor and the lessons you taught me about always working hard and doing my best.

To my brother, you've been my partner in crime and it has been a complete joy to get to become an adult with you. Your strength inspires me and your sense of humor is the best around. Plus, your memes are on point.

To all the furry creatures in my life, but especially Theo and Arlo, thank you for the endless love and cuddles.

And finally, to the love of my life, my partner, and my best friend, James. I could fill a whole new dissertation with everything you have meant to me over the past few years. You pushed me when I was at my lowest and helped me see the joy and excitement in my work when I could not. You have a beautiful ability to make the people around you want to be the best versions of themselves and every day with you is somehow magically better than the last. I cannot wait to be Dr. and Mr.

FUNDING SUPPORT

This dissertation was funded in part by a grant from the National Institutes of Health, USA (NIH grant 5T32ES007141-34). Funding was also provided by the Johns Hopkins University Education and Research Center for Occupational Safety and Health (ERC). ERC training grant funding comes from the National Institute for Occupational Safety and Health (NIOSH), under Grant No. 5 T42 OH 008428. This project was also funded through NIOSH under Grant No. R21 OH 010661. Supported in part by resources provided by NIH/NIDDK GI Core Center, "Hopkins Digestive Diseases Basic & Translational Research Core Center P30-DK089502." Funding was also provided by The Osprey Foundation.

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CHAPTER ONE:

Introduction

Diarrheal illnesses remain a significant global health burden, causing approximately 1.6 million deaths per year, a third of which are in children under the age of five (1,2). The majority of acute gastrointestinal illnesses (AGI) are caused by human noroviruses (HuNoVs) (3). Each year, HuNoVs cause an estimated 685 million illnesses and cost the global economy $64.5 billion (3-5). Approximately 200,000 people die of HuNoVs annually, and about a quarter are children under the age of five (5). In the US alone, 1 in 20 people become infected with HuNoV each year and this high rate of illness leads to significant societal burdens. This includes direct strain on healthcare systems, loss of productivity and reduced quality of life due to illness, and rare, but severe, long term complications arising from chronic cases (6).

HuNoVs are members of the *Caliciviridae* family and are thought to cause illness exclusively in humans (7). Typical symptoms of HuNoV infection are nausea, vomiting, and diarrhea which are typically of short-duration (2-3 days) and self-limiting in immunocompetent hosts (7). Transmission of HuNoVs occurs through the fecal-oral route and these viruses are highly infectious (6,8). HuNoVs are stable in the environment for up to weeks or even months, are shed in large numbers by both symptomatic and asymptomatic infected hosts, and very few

virions are required for infection (9-13). HuNoVs encompass multiple viral strains that differ slightly in structure but retain basic similar characteristics (14). Namely, HuNoVs are small (~27nm diameter), spherical, non-enveloped viruses with a T=3 icosahedral structure formed by repeats of one major (VP1) and one minor (VP2) structural proteins (15). HuNoV genomes consist of single stranded positive sense RNA, ~7.7 kb long, and contain three open reading frames - ORF1 encodes nonstructural proteins, while ORF2 and ORF3 encode the structural proteins VP1 and VP2 (16).

HuNoVs are classified by a genogroup and genotype number that relate to slight structural variations. The two main genogroups that cause human illness are one and two - represented as GI and GII, respectively (17,18). Genotype is represented by a number after genogroup and a range of genotypes cause illness, with new types continuously emerging as the virus evolves (19). A third level of HuNoV categorization is strain name, usually related to the geographic location of first isolation. For example, the main epidemic strain of HuNoV that is currently in global circulation is GII.4 Sydney - genogroup two, genotype four, strain Sydney (20,21).

In addition to their high transmissibility, HuNoVs pose unique challenges for research and detection. The most significant of these challenges is the historical

lack of a reproducible cell culture system for propagating infectious HuNoVs (22,23). The first documented case of HuNoV was from an outbreak of AGI in a school in Norwalk, Ohio, USA in 1968 and the strain was identified four years later in 1972 (24). However, HuNoVs have remained resistant to in vitro culture for over four decades (25,26).

The lack of a reproducible cell culture system for HuNoV had profound impacts on research and monitoring efforts (27,22,23). Molecular tools that detect the presence of HuNoV RNA remain the most common tool for identifying and working with HuNoVs (28). The most common of these molecular tools is reverse transcription-quantitative polymerase chain reaction (RT-qPCR) using primers and probes that target the ORF1-ORF2 capsid junction or the RNA dependent RNA polymerase in the viral genome (29). RT-qPCR offers many benefits for detecting HuNoVs - the method is relatively rapid to perform (3-4 hours), very sensitive, and easily quantifiable (30,31). The significant caveat to RT-qPCR and other molecular approaches for detecting HuNoVs is the inability to distinguish infectious particles from non-infectious segments of RNA (22). Extensive literature has been dedicated to understanding the relationship between molecular measurements of HuNoV RNA and the presence of actively infectious HuNoVs (32,22,33). Alternative methods that are thought to better approximate infectivity have been suggested, but besides inoculating human volunteers

(10,34), a reproducible cell culture remains the only way to confirm the presence of infectious HuNoV (35,32,36,37).

In 2016, the first reproducible HuNoV cell culture system was developed (38). This represented a momentous stride in HuNoV research and opened the door for a slew of new ways to study HuNoVs. The HuNoV cell culture system uses human intestinal enteroids (HIEs) which are three-dimensional spheroids that recapitulate the epithelium of the human small intestine (38). HIEs are multicellular, differentiated, physiologically active cultures that contain all of the main intestinal epithelium cell types including $LGR5^+$ stem cells, Paneth cells, enteroendocrine cells, enterocytes, and goblet cells (39-41). In particular, HIEs used to grow HuNoVs are from jejunal biopsies as this is thought to be the major area of HuNoV replication in the body (38). HIEs are established by isolating crypt cells from a human jejunal biopsy that can then produce multiple spheroids. HIEs are not immortal, but can be sequentially passaged and maintained for multiple months or even years (42,38,39).

Infection of HIEs with HuNoVs requires breaking spheroids into a single cell suspension and seeding this suspension on a basement membrane to generate monolayers (42,38). These monolayers are differentiated to recapitulate mature enterocytes, enteroendocrine cells, and goblet cells, then HuNoV suspensions

are added directly to monolayers (43). HIE monolayers are not readily quantifiable as they do not form plaques or other easily identifiable signs of cytopathic effect that are used to quantify viruses in other cell culture systems (44,38,45). Instead, HIEs must be infected in duplicate and then viral RNA is extracted from one plate at one hour post infection (hpi), while the second plate grows for 72 hpi before viral RNA extraction (43). The RNA extracted at 1 and 72 hpi is quantified with RT-qPCR and changes in HuNoV genome equivalents (GE) are compared between the two time points. An increase in GE is indicative of actively infectious HuNoVs (38).

Though HuNoV cultivation in HIEs is a major scientific breakthrough, this system is subject to numerous limitations. HuNoV growth in HIEs can be highly variable both within and across strains (42,45). The need for RT-qPCR complicates the ability to obtain quantitative measures of growth from HIEs. Additionally, HIEs are extremely complex systems that require a significant level of training, labor, and monetary resources to establish and maintain (44,43). The reagents to test a single HuNoV sample in HIE culture cost approximately $36 and the entire process from start to finish requires a minimum of three weeks. These challenges with the HuNoV cell culture system affect all applications of the method. However, the drawbacks of the HIE system become significantly more pronounced when the goal is to use HIEs to attempt to grow the low titers of

HuNoV recovered from the environment, either for monitoring, risk assessment, or to test inactivation methods.

Environmental monitoring of HuNoVs could be revolutionized by the development of the HIE cell culture method. The ability to identify infectious HuNoVs in the environment can help target areas for intervention, allow for more accurate testing of inactivation methods, and can improve risk assessments (46-48). Risk assessments, also referred to as quantitative microbial risk assessments (QMRA), are a valuable tool in translating microbial monitoring data into public health impacts. Briefly, QMRA consists of four steps that require identification of the hazards, precise description of exposure, quantification of dose-response, and integration of quantitative data to model risk values for an exposure scenario (49). HuNoV QMRAs have been significantly hampered by the lack of data on HuNoV presence and transmission (50-52). The HIE cell culture offers a new data source that could help improve QMRA and risk prediction for HuNoVs.

The goal of this dissertation was to lay the groundwork for use of the HuNoV HIE cell culture method in environmental monitoring scenarios and ultimately in QMRAs. The steps taken to achieve this goal can be summarized in three primary research objectives:

1. Measure the impact of key experimental variables on HuNoV growth in HIE cells and establish a set of recommendations for moving forward with monitoring applications.

2. Determine the conditions necessary to grow surface-recovered HuNoV in HIE cells and characterize recovery of infectious HuNoV.

3. Develop a QMRA that can serve as a template for integrating HuNoV cultivation data in the future.

Each research objective is presented as a standalone chapter that represents a manuscript in preparation for peer review.

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CHAPTER TWO:

Optimizing human intestinal enteroids for environmental monitoring of human noroviruses

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Abstract

Human noroviruses (HuNoV) are the leading cause of gastrointestinal illness and environmental monitoring is crucial to prevent HuNoV outbreaks. The recent development of a HuNoV cell culture assay in human intestinal enteroids (HIEs) has enabled detection of infectious HuNoV. However, this complex approach requires adaptation of HIE to facilitate HuNoV replication from environmental matrixes. Integrating data from 200 experiments, we examined six variables: HIE age, HIE basement membrane compounds (BMC), HuNoV inoculum processing, HuNoV inoculum volume, treatment of data below limit of detection (LOD), and cutoff criteria for determining positive HuNoV growth. We infected HIEs with HuNoV GII.4-Sydney positive stool and determined $1.4x10^3$ genome equivalents per HIE well were required for HuNoV replication. HIE age had minimal effect on assay outcomes. LOD replacement and cutoff affected data interpretation, with lower values resulting in higher estimated HuNoV detection. Higher inoculum volumes lead to minimal decreases in HuNoV growth, with an optimal volume of

250uL facilitating capture of low concentrations of HuNoVs present in environmental isolates. Processing of HuNoV inoculum is valuable for disinfection studies and concentrating samples, but is not necessary for all HIE applications. This work enhances the HuNoV HIE cell culture approach for environmental monitoring. Future HIE research should report cell age as days of growth and should clearly describe BMC choice, LOD handling, and positive cutoff.

1 Introduction

Human noroviruses (HuNoVs) are the leading cause of acute gastroenteritis globally and cause approximately 200,000 deaths and $64 billion in economic losses each year (1,2). Due to high transmissibility and persistence, HuNoVs in the environment pose a significant infection risk which makes accurate monitoring crucial for prevention and control (3,4).

Traditionally, HuNoVs resisted culture efforts (5) and could only be detected with molecular methods that measure viral RNA (6-10). These approaches frequently use reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and values can be converted to a measure of HuNoV genome equivalents (GE) using an internal standard with a known amount of RNA (11,12). Though molecular approaches are fast, sensitive, and easily quantifiable, the detection of HuNoV

RNA is not necessarily indicative of infectious virus (13,14). Additionally, RT-qPCR assays can be subject to reaction inhibition as a result of organic matter present in samples (15).

The inability to measure infectious HuNoV virions can hamper public health interventions as the relationship between HuNoVs detected via RT-qPCR and infectivity in a human host is not clear (9). Several approaches to improve nucleic acid amplification correlation with viral infectivity have been reported (16-20). A reproducible cell culture infectivity model remains the definitive way to confirm the presence of infectious HuNoV (8,9). Identifying infectious HuNoV by cell culture can be a direct correlation to human health outcomes. An infectivity model presents the opportunity to develop more accurate risk assessments, predict health outcomes based on monitoring data, and conduct viral inactivation experiments that better capture true reduction of infectivity (21,22).

An approach that has been successfully used to cultivate HuNoV is human intestinal enteroid (HIE) cell culture (23). HIEs, also termed “mini-intestines,” are three-dimensional polarized structures that recapitulate the human intestinal epithelium (24,25). For growth of HuNoV, crypt cells are isolated from a human jejunum biopsy to generate jejunal HIEs as the second section of the small intestine is considered the main site of HuNoV replication (26). HIEs require the

addition of growth factors, including Wnt3a, R-spondin, and Noggin, to stimulate the development of crypt cells into multi-cellular spheroids that contain the major cell types found in the human intestinal epithelium (24,27). HIEs mimic major aspects of normal intestinal epithelial physiology, including electron transport and cell lifespan, and demonstrate key pathophysiological responses to pathogen infection (28,25).

HIEs are maintained as 3D cultures prior to processing the HIEs for infection with HuNoV (26,29). Preparation for infection involves disrupting the HIEs into a single cell suspension, subsequent seeding of cells as monolayers in 96 well tissue culture plates, and inducing differentiation by removal of Wnt3a, which leads to the development of mature absorptive enterocytes and secretory cells (26,25,29). These differentiated HIE monolayers can then be infected with HuNoV (26). The HIE method shows promise in bridging the gap between measurements of RNA and the presence of actively replicating HuNoV virions by mimicking the physiological processes of the gut (30,31,26).

In HIEs, HuNoV growth is measured as the fold increase in HuNoV GE between 1 and 72 hours post infection (hpi) (26). A cutoff value for fold-increase in GE can then be set to classify samples as positive or negative for replication of infectious HuNoV. The ability to culture HuNoV in vitro has important implications for

vaccine research, clinical detection, and testing inactivation methods (32,33,30,34,23,35-39). An important application of HIE cell culture is growing HuNoV recovered from food, water, air, and fomites. The isolation and detection of HuNoV RNA recovered from environmental isolates is frequently used to monitor cleaning efficacy and determine potential interventions that are needed to protect human health (40-43). However, environmental monitoring has different technical needs than other applications of the HIE system (44,45,10,46). First, quantitative values for infectious HuNoV need to be reproducible and meaningful. In monitoring applications, infectious HuNoV detection must be reflective of input HuNoV and samples with similar input should yield similar measures of infectious HuNoV (15,43). Second, qualitative cutoffs to determine positive versus negative samples must be robust and consistent. In monitoring, qualitative measures of presence/ absence are frequently used and these measures must accurately reflect underlying presence of HuNoV (44,43). And third, methodologies should be standardized to ensure comparable values across research groups (47). Choices of reagents, handling of HIE cells and HuNoV inoculum, and data interpretation and presentation should be done in a way that allows for ease of replication and comparison across studies (44,47).

Refining the HIE cell culture method to address the needs of environmental monitoring is hindered by the complexity in maintaining and infecting HIEs with HuNoV (31,23). The HuNoV HIE culture method requires numerous reagents,

including specialized collagen matrix and growth media, multiple laborious steps, including manual trituration of cells, and significant financial investment (23). This study reports on key aspects for HuNoV cultivation in HIE with a focus on developing approaches for environmental samples. The generated data were subsequently used to develop best practices for propagating infectious HuNoV in HIEs.

2 Methods

Figure 1 outlines the method for growing human norovirus in HIE. We measured infectivity of HuNoV isolated from norovirus-positive human stool samples in HIE using previously described methods (30,26). Six key experimental variables were addressed to examine impacts on HuNoV growth:

HIE culture variables:

1. HIE cell age
2. BMC for seeding HIE monolayers

HuNoV suspension variables:

3. Processing method for HuNoV suspensions
4. Volume of HuNoV suspension added to HIE

Data processing variables:

5. Handling of values that fall below the limit of detection (LOD)
6. Cut off value for determining if a sample contains infectious HuNoV

2.1 Human Norovirus Stool Samples & Processing

We tested four HuNoV GII positive stool samples from adult and pediatric patients; samples were genotyped based on the capsid region (48) (Table 1). All samples were diluted to 10% in sterile phosphate buffered saline and filtered through a 0.45μm filter. A subset of the pediatric GII.4 Sydney stool sample was further processed with one of three methods: Vertrel XF (DuPont, Wilmington, DE); Vertrel XF plus 0.45μm filtration; or Vertrel XF, 0.45μm filtration, and sucrose cushion ultracentrifugation, as previously reported (49). Briefly, equal parts 10% stool filtrate and Vertrel XF were homogenized on ice and the emulsified mixture was centrifuged for 15 min at 4000 x g; supernatant was recovered and used as “Vertrel” labeled stool suspension. A subset of Vertrel suspension was then passed through a 0.45 μm filter and resulted in “Vertrel and filtered” stool suspensions. The highest processing step involved purification of Vertrel and filtered suspensions with sucrose cushion ultracentrifugation. The Vertrel and filtered HuNoV suspension was overlaid on a sterile-filtered 20% sucrose solution in an Ultraclear centrifuge tube (Beckman, Brea, CA) and centrifuged for 3 h at 95,000 x g; this process was repeated using an additional Vertrel and filtered HuNoV suspension before suspending the resulting pellet. The final suspension was defined as a “sucrose” HuNoV suspension. Samples were portioned and immediately stored at -80°C until time of testing. Portions were used for a maximum of three individual experiments and were limited to no

more than three freeze-thaw cycles.

2.2 Human Intestinal Enteroid Culture

We maintained a secretor-positive jejunal HIE culture (J2 line), kindly provided by Mary Estes (Baylor College of Medicine, Houston, TX), as undifferentiated three-dimensional (3D) (i.e. spheroid) cultures. This line has been previously used to grow HuNoV and human rotavirus (26,28). Human IntestiCult media (STEMCELL Technologies Inc., Vancouver, Canada) was used as complete media with essential growth factors to propagate HIEs; complete media without growth factors (CMGF-) was prepared as previously described (26). Cultures were maintained at 37°C in 5% CO2 in 24-well cell culture plates. After 7 days of growth, 3D cultures were either split 1:2, archived in liquid nitrogen (LiN_2), or dissociated to a single cell suspension and plated 1:2 as an undifferentiated monolayer in a 96-well cell culture plate. HIE monolayers were seeded on Matrigel (Corning, Corning, NY) or human Collagen IV (Sigma-Aldrich, St. Louis, MO), and grown for two days in IntestiCult supplemented with 10 µmol/L Y-27632. Monolayers were subsequently differentiated for 5 days prior to infection with media prepared by Johns Hopkins Conte NIH/NIDDK Digestive Diseases Basic and Translational Research Core Center, as previously described (50,28). Cell age was measured in terms of passage number, days of continuous growth, and length of time archived in LiN_2. Passage number was not reflective of HIE

freeze-thaw cycles. Days of continuous growth represent the number of days between removal of HIEs from LiN_2 archive and subsequent infection with virus. In some instances, propagated HIEs were obtained directly from other laboratories within Johns Hopkins University. In these instances, due to the lack of propagation history, receipt of HIE cultures by our lab personnel were considered day 1 of growth.

2.3 Human Norovirus Infection Experiments

Confluent HIE monolayers were infected apically after 5 days of differentiation, in duplicate, with processed HuNoV virus supplemented with 500 μM of glycochenodeoxycholic acid (GCDCA; Sigma-Aldrich, St. Louis, MO). After 1 hour of incubation at 37°C in 5% CO2 to allow viral attachment, the supernatant was removed and monolayers were washed three times with CMGF-. For each set of infections, after the third wash, one monolayer was immediately frozen at -80°C and the second was grown at 37°C in 5% CO2 for 72 hours post infection (hpi). Following the 72-hour incubation, the supernatant and monolayer cells were frozen at -80°C. We extracted RNA from 1 hpi and 72 hpi monolayer cells and supernatants with the Direct-zol RNA miniprep kit (Zymo Research, Irvine, CA). HuNoV RNA copies were measured with RT-qPCR using the QuantiTect Probe RT-PCR Kit (Qiagen, Hilden, Germany) with COG2 primers and the RING2P probe targeting the ORF1-ORF2 junction (51).

In order to calculate a quantitative HuNoV GE we developed a standard curve using in vitro RNA transcripts derived from plasmid pNoV/MD145, kindly provided by Michael Kulka (FDA, Silver Spring, MD, USA), which contained a full-length synthetic cDNA copy of a HuNoV GII strain (12). Based on 14 runs of seven dilutions in duplicate, the RT-qPCR limit of detection (LOD) was determined to be 44.3 viral GE/ 5uL, as calculated using the discreet threshold method (47).

2.4 Statistical Analyses

Statistical analyses were performed in Stata 13 and R 3.6.1 (StataCorp, 2013; R Core Team, 2019). We use the term HuNoV growth to refer to the fold increase in HuNoV GE between 1 and 72 hpi in HIEs. Unless otherwise stated, samples were considered negative for infectious HuNoV if the fold increase was less than five. Values below the RT-qPCR LOD (44.3 GE/ 5uL) were replaced with the LOD value unless stated otherwise.

The Kruskal-Wallis one-way ANOVA on ranks test (KW) was used to examine the relationship between single input variables and fold increase as a continuous outcome; the Kruskal-Wallis test was chosen as the data were non-parametric. Age of HIEs was treated as a categorical variable due to the pseudo-continuous nature of age values. HIE passage numbers were aggregated to categories of

20-29, 30-39, 40-49, and 50-59; HIE days of growth were aggregated to 0-49, 50-99, 100-149, and 150-199; HIE days in archive were aggregated to 0-99, 100-199, 200-299, and 300-399. For many variables, we aimed to control for input virus when measuring statistical differences. To achieve this, significance was tested with binomial logistic regression (BR) for binary outcomes and linear regression (LR) for continuous outcomes. All continuous outcomes were log transformed prior to regression analysis.

3 Results

3.1 Selection of HuNoV Strain

We screened four 10% stool suspensions containing HuNoV to determine their ability to replicate in HIEs (Table 1). Two samples replicated in the HIE system – a pediatric GII.4 Sydney and an adult GII.4-16 recombinant (data not shown). The increase in HuNoV GE between 1 and 72 hpi for the two virus strains is shown in Figure 2. No significant difference was observed in increase in HuNoV at 72 hpi (KW p=0.29). Ten of 38 GII.4-16 recombinant experiments were positive for infectious HuNoV, while 18 of 33 GII.4 Sydney experiments were positive. Due to the lower detection rate of infectious norovirus in GII.4-16 recombinant experiments, the pediatric GII.4 Sydney virus was used in all subsequent experiments, unless stated otherwise.

3.2 Age of HIE Cells

Three measures were used to examine the relationship between age of HIE cells and HuNoV growth (Figure 3). When controlling for input virus, HIEs at passage 40 to 49 were associated with a 2% increase in odds of detecting HuNoV, compared to passage 20-29 (BR p=0.04); no other passage category was associated with an increase in detecting infectious HuNoV (BR p-values>0.05). When HIE age was measured as days of growth, there was no association with detection of infectious HuNoV (BR p-values>0.9). Similarly, no association was observed between category of days archived in LiN_2 and qualitative detection of infectious HuNoV (BR p-values>0.05). HuNoV growth was observed at the maximum value for all three measures of age: passage 58, 187 days of continuous growth, and 334 days archived in LiN_2.

3.3 Basement Membrane Compound

The relationship between HuNoV growth and HIE monolayer BMC is shown in Figure 4. Fifty-four percent (75/140) of Matrigel seeded HIE monolayers were positive for infectious HuNoV, while 32% (7 of 22) of collagen experiments were positive. The proportion of samples positive for infectious HuNoV were not significantly different between the two membranes when controlling for input virus (BR p=0.1). However, when controlling for input virus, HIE monolayers seeded with Matrigel had a significantly higher measured fold increase in HuNoV GE

between 1 and 72 hpi, compared to HIE monolayers seeded with Collagen IV (LR p = 0.03).

3.4 Titer of Input HuNoV

Prior to investigating the role of HuNoV viral suspension variables in growth in HIEs, we measured the relationship between input HuNoV GE and measured growth for HIEs using 100µL inoculation volumes of 0.45µm filtered virus ("baseline" experiments denoted with grey points in Figures 5 and 6). An increase in input HuNoV was significantly associated with a higher likelihood of detecting infectious virus (BR p=0.03) and with an increase in HuNoV growth as measured by fold increase (KW p = 0.03). No growth of HuNoV was observed at input values below 1.4×10^3 GE/ well (data not shown).

3.5 HuNoV Inoculum Processing

The impact of different viral inoculum processing steps on HuNoV growth, compared to baseline experiments, is shown in Figure 5. No significant association was observed between proportion of samples positive for HuNoV across the three additional processing steps when compared to baseline (BR p>0.3). When controlling for amount of input HuNoV, there was no association between HuNoV growth and processing method (LR p>0.3)

3.6 HuNoV Inoculum Volume

The impact of infecting HIEs with different volumes of HuNoV suspension on HuNoV growth are shown in Figure 6. Compared to a 60% positive detection rate for 100µL samples (70/116), the detection rate was 50% for both 200µL (6/12) and 250µL (2/4) infections and 75% for 300µL infections (6/8). When controlling for amount of HuNoV GE, HIEs inoculated with 200µL of HuNoV stool suspension had reduced odds of positive detection of infectious HuNoV, compared to 100µL (BR $p=0.009$). However, volumes higher than 200µL were not associated with decreased detection of HuNoV (BR p-values>0.1).

3.7 Limit of Detection

The three methods for replacing values below the limit of detection resulted in a significant difference in proportion of samples positive for HuNoV (BR $p<0.0001$). The highest percent detection of infectious HuNoV was observed when below LOD values were replaced with half of the LOD (61%, 71/116), followed by replacement with the LOD (60%, 70/116), and then dropping values below the LOD (33%, 38/116). When only positive experiments were then considered in each category, fold increase in HuNoV GE in HIEs across the three methods was not significantly different (Figure 7, KW $p=0.37$).

3.8 Positive Fold Cut-Off

We examined the impact different cut-off values for fold increase in HuNoV GE between 1 and 72 hpi to determine if samples contained infectious HuNoV. Three cut-off values were selected - greater than or equal to 1, 5 (baseline), and 10 (Figure 8). The percent of samples positive for infectious HuNoV were not significantly different across the three cut-off values and ranged from 65% for a fold cut-off of ≥1 (75/116) to 57% for a fold cut-off of ≥10 (68/116) (BR p≥0.3).

To examine how differences in fold cutoff may affect data interpretation, we analyzed the relationship between fold increase for positive samples and the amount of input virus, as this association is well documented in the literature. For each fold cutoff, there was a significant association between input HuNoV and fold increase for positive experiment as measured by the Kruskal-Wallis one-way ANOVA on ranks test (p-values<0.03).

4 Discussion and Conclusions

The ability to grow HuNoV in HIEs is a significant advancement for HuNoV research (31,23). The HIE system enables the definitive detection of infectious HuNoV, which has a profound impact on vaccine research, clinical monitoring, and environmental detection for HuNoV (32,33,30,34,23,35-38,52,39). Of particular interest is environmental monitoring applications of the HIE cell culture

system for HuNoV. Environmental presence of HuNoV is frequently used to measure efficacy of interventions and assess public health risk (40-43). Monitoring applications can greatly benefit from the integration of cell culture to detect infectious HuNoV (53). This would allow for more accurate predictions of health outcomes and could prevent the unnecessary expense of resources to treat environmental sources that may contain HuNoV RNA but no actively infectious HuNoV.

To this end, we sought to further develop and codify the HIE cell culture system to facilitate HuNoV environmental monitoring applications. Methodological details can vary across research groups and include components of the HIE culture itself, handling of the HuNoV suspensions to be tested, and data processing decisions that have yet to be systematically evaluated (Table 2). We selected some of these methodological details to examine the effects on qualitative and quantitative measures of infectious HuNoV and translated these data into a list of recommendations (Table 3). For the HIE system, qualitative measurements describe the number of samples considered positive for infectious HuNoV, while quantitative measurements reflect the increase in HuNoV GE in the HIE system, usually represented as fold increase.

Prior to testing experimental variables, we tested four HuNoV positive stool

samples to identify the best candidate for growth in HIEs. The most successful growth was from a pediatric stool sample that contained HuNoV GII.4 Sydney. This is consistent with prior reports that GII viruses and stool from pediatric patients grow best in the HIE system (30,23).

4.1 HIE Culture Variables

The first HIE cell culture variable we examined was cell age. Previous work with HuNoV in HIEs either did not report this information or included HIE age as passage number, which represents the number of times a line has been split (32,54,30,34,26,35,37,38,52,39). We analyzed HIE age data three ways: as cell line passage number, as days of continuous growth in the lab prior to infection with HuNoV, and as number of days the line had been archived in LiN_2 (Figure 3). We found that we were able to observe successful growth up to passage 58, which is significantly higher than previous studies that report a maximum passage of 31 (54,30,52). An increase in passage number was only associated with an increase in the percent of samples positive for HuNoV for HIEs at passage 40-49; while no relationship was found for days of continuous growth or days archived. This discrepancy illustrates that passage number may not be ideal as a standalone measure of cell age. Each passage equates to roughly one week, but passage number is not directly tied to any age, as cell lines can be frozen and unfrozen without any change in passage number. The inconsistent

association between cell age and HuNoV replication has been observed by others where a 3 $\log_{10}$ difference in HuNoV replication in HIEs across a four-year period was observed, but no apparent relationship between time cultured and resulting HuNoV replication (23).

The second HIE associated variable we examined was the BMC used to stabilize monolayers seeded onto 96 well tissue culture plates. BMCs are comprised of protein matrices that are deposited beneath epithelia and form sheets that provide mechanical stability and can influence cell shape and proliferation (55). There is no consensus in the literature on choice of BMC for HIEs to cultivate HuNoV. Previous methods for growing HuNoV in HIEs report the use of either Collagen IV (32,30,34,38,52,29) or Corning Matrigel (26,35,37). We found that the two different BMCs resulted in similar qualitative measures of infectious HuNoV (Figure 4), but when accounting for amount of input virus, more HuNoV growth was observed in HIE monolayers seeded with Matrigel. A low sample size was used for Collagen IV experiments, compared to Matrigel, as we switched all experiments to Matrigel after observing successful growth. As such, it is possible that the two methods are comparable. However, we maintain our suggestion of Matrigel as a BMC for environmental monitoring applications because per experiment, the cost of Matrigel is about 30% lower than that of Collagen IV, and cost reduction is an important consideration to increase the feasibility of HIEs for regular monitoring applications of HuNoV.

4.2 HuNoV Infection Inoculum Variables

Multiple research groups have determined that higher input viral titer leads to more successful growth of HuNoV in HIEs and we confirm these findings (30,23). Our work showed that the minimum HuNoV dose required to measure growth in HIEs is 1.4 x 10^3 GE/ well (Figures 5 and 6), consistent with previous reports (30).

The first HuNoV inoculum variable we studied was processing of HuNoV stool prior to infection in HIEs. Currently, no variability in processing method for HuNoV stool is observed in the literature - all studies report the use of 10% stool filter through either a 0.22μm or a 0.45μm filter (32,54,30,26,23,52,39,29). Our interest in HuNoV stool suspension processing was driven by data that indicates more highly processed samples, with less organic load, are desirable for disinfection studies (49). As disinfection studies are an important application of the HIE culture system, it was important to test alternative methods for processing HuNoV stool samples (30). A reduction in organic load in viral inoculum has an important effect on disinfection kinetics and allows for a more accurate measure of both disinfectant residual and true efficacy of the disinfectant on HuNoV (56,57). Additionally, HuNoV recovered from the environment is likely to be very low titer which necessitates concentration

methods (58-60). However, historical efforts to cultivate HuNoV indicated that higher purity samples may actually resist growth due to the absence of necessary components present in stool (5). Our results show that this is not the case and that high purity HuNoV samples processed with a combination of Vertrel XF, additional filtration, and/ or sucrose cushion ultracentrifugation do not replicate differently in HIEs.

We were also interested in the volume of HuNoV stool suspension that was used to inoculate HIE monolayers. Previous HuNoV HIE cell culture work consistently used 100µL of infection volume, or did not report this variable at all (32,54,30,34,26,37-39,29). Environmental samples are frequently low titer and the ability to test higher volumes in HIE culture may have value in improving detection of these types of samples (58,60,10). We tested infection volumes of 200µL, 250µL, and 300µL (maximum volume of well) and found a slight decrease in detection of infectious HuNoV when using 200µL, but no association for the other volumes (Figure 6). It is important to note that statistical measures for volume are likely subject to errors with small sample sizes in higher volume categories, compared to baseline 100µL tests. The 300µL infection experiments had a higher rate of detection (75%) of infectious HuNoV compared to 100µL experiments (60%). We suggest the use of a higher infection volume for environmental monitoring because the ability to test larger sample volumes can allow for improved detection in low titer samples. A final infection volume of

250µL is suggested because 300µL is close to the maximum capacity of wells and could lead to spills, sample loss, or contamination.

4.3 Data Processing Variables

Finally, we investigated how different data processing decisions impact results from growing HuNoV in HIEs. The first of these decisions was the method used to replace data points that were below the RT-qPCR assay LOD. Environmental monitoring is likely to produce many values that fall below the LOD of the RT-qPCR assay due to low viral titer in the environment (40,58-60). Previous literature has indicated that the choice of LOD handling can significantly impact outcomes, but no consensus on the most appropriate method exists in the literature (61,62). We chose to investigate methods that replaced below LOD values uniformly, either with the LOD value, half the LOD value, or with zero, effectively dropping the sample. We did not investigate any methods for predicting below LOD values with a model as this may be too computationally intense for regular monitoring applications (61). Based on our results, we do not suggest dropping below LOD values as this can lead to a significant underestimation of infectious HuNoV. However, the difference between using the LOD value versus half the LOD is less clear and resulted in similar measures of HuNoV. This relationship warrants further investigation and until stronger rationale is available, we suggest explicitly stating the LOD replacement method

in the Methods when using HIEs for HuNoV detection and analysis.

The second data handling decision that we investigated was the choice of cutoff for fold increase in HuNoV GE between 1 and 72 hpi to determine if a sample contained infectious HuNoV. Fold cutoff values vary in the literature from greater than or equal to 1 up to 10, with some studies not reporting a cutoff at all and presumably including all samples with a positive fold increase (54,30,34). For most of this work we chose a positive fold cutoff of greater than or equal to five as this was the middle of previously reported values. We found that the choice of fold cutoff did not lead to statistical differences in the percent of samples identified as positive for HuNoV (Figure 7). However, final percent positive samples ranged by 8% between the lowest cutoff, ≥ 1, and the highest cutoff, ≥ 10. This difference could be significant in monitoring scenarios as high cutoff values could miss up to 8% of true positives, while low cutoff values could lead to more false positives.

To examine how differences in fold cutoff may affect data interpretation, we analyzed how different cutoff values impacted the relationship between fold increase for infectious HuNoV positive samples and the amount of input virus, as this association is well documented in the literature (30,23). We found that for all three values of fold cutoff there was a statistically significant positive association

between viral input titer and measured fold increase. This indicates that differing fold cutoffs may affect qualitative measures of infectious HuNoV, but likely do not alter measured associations between variables. For environmental monitoring applications of the HIE system, the lowest fold cutoff will result in data that is the most protective of human health, but may lead to the decision to dedicate limited resources to addressing what ultimately may be false positive measures of infectious HuNoV. Due to this existing uncertainty, the use of multiple fold cutoffs remains ideal to account for potential false positives and negatives.

This work addressed multiple methodological challenges in growing HuNoV using an HIEs approach, with specific attention towards applying the method to growing HuNoV recovered from the environment. Accurate measurement of infectious HuNoV in the environment, including food, water, and fomites, is a crucial first step in improving risk assessments for HuNoV infections in various settings. Additionally, these methodological refinements also improve the use of the HIE system for growing HuNoV samples that have been subject to inactivation or disinfection methods. Recommendations from this work (Table 3) serve as a foundation for future application of the HIE system to measuring infectious HuNoV recovered from the environment. These recommendations can guide future studies and form a blueprint for continued improvement of the HuNoV HIE cell culture method.

Acknowledgements

The authors would like to thank Samantha Wales from FDA CFSAN and Sunny Lee at Johns Hopkins School of Medicine for technical assistance.

Tables

Table 2.1 Human norovirus (HuNoV) positive fecal samples tested for replication in human intestinal enteroid (HIE) monolayers

HuNoV Genotype	RNA copies/ 100µL Undiluted Stool Suspension	Patient age group*	Collection date
GII.4-16 Recombinant	2.0×10^7	Adult	Jan 2017
GII.4 Sydney	3.22×10^7	Pediatric	Jan 2018
GII.2	6.43×10^6	Adult	Jan 2017
GII.4 Sydney	6.02×10^4	Adult	Dec 2017

*Adult >5 years of age, pediatric <5 years of age.

Table 2.2 Summary of experimental variables investigated for their impact on human norovirus (HuNoV) growth in human intestinal enteroids (HIEs)

Variable	Description	Options for Handling or Addressing Variable	Treatment of Variable in Previous Literature
Age of HIE cells	Age of HIE cells used to seed monolayers for HuNoV growth	1. Report passage number which reflects how many times an HIE line has been split 2. Report days of continuous cell growth, independent of passage number 3. Report data related to archiving of HuNoV in liquid nitrogen; either as number of freeze-thaw cycles or amount of time frozen	Most studies do not provide information on HIE cell age (32,34,26,35,37-39) and those that do only report passage number, with growth occurring in up to passage 30 (54,30,52)
Basement Membrane Compound	Media used to seed HIEs as a monolayer prior to HuNoV infection	1. Corning Matrigel 2. Collagen IV	Previous methods for growing HuNoV in HIEs report the use of both Collagen (32,30,34,38,52,29) and Matrigel (26,35,37); no explanation is offered for the choice between the two

Viral Processing Method	Processing method for HuNoV positive stool suspensions prior to HIE infection	1. Prepare 10% stool suspension and filter through a 0.45 µm or 0.22µm filter 2. Use further processing steps, such as Vertrel XF or sucrose cushion centrifugation, that concentrate samples and reduce organic matter	Most studies use 0.45 µm or 0.22µm filtered 10% stool suspensions (32,54,30,26,23,52,39,29)
Infection Volume	Volume of HuNoV positive stool suspension added to each monolayer well	1. Infect with the standard volume from initial HIE reports: 100 µL 2. Increase the volume to test more sample, up to the maximum well volume of 300 µL	Most studies report using 100µL infection volumes (54,30,37,39,29); while some do not provide this information (32,34,26,38)
LOD Handling	Method for handling data that falls below the RT-qPCR limit of detection (LOD)	1. Drop all values below the LOD 2. Replace all LOD values with half the LOD or with the value of the LOD itself	Below LOD values are most frequently replaced with half of the LOD (54,30,23,37) or a method for handling below LOD values is not stated (32,38,52,39)

Fold Cutoff	Fold change in HuNoV genome equivalents (GE) between 1 and 72 hours post infection (hpi) in HIEs used to identify positive samples	A range of numbers can be selected for this value	Reported fold cutoffs include >1 fold (30), >2 fold (34), and >10 fold (54)

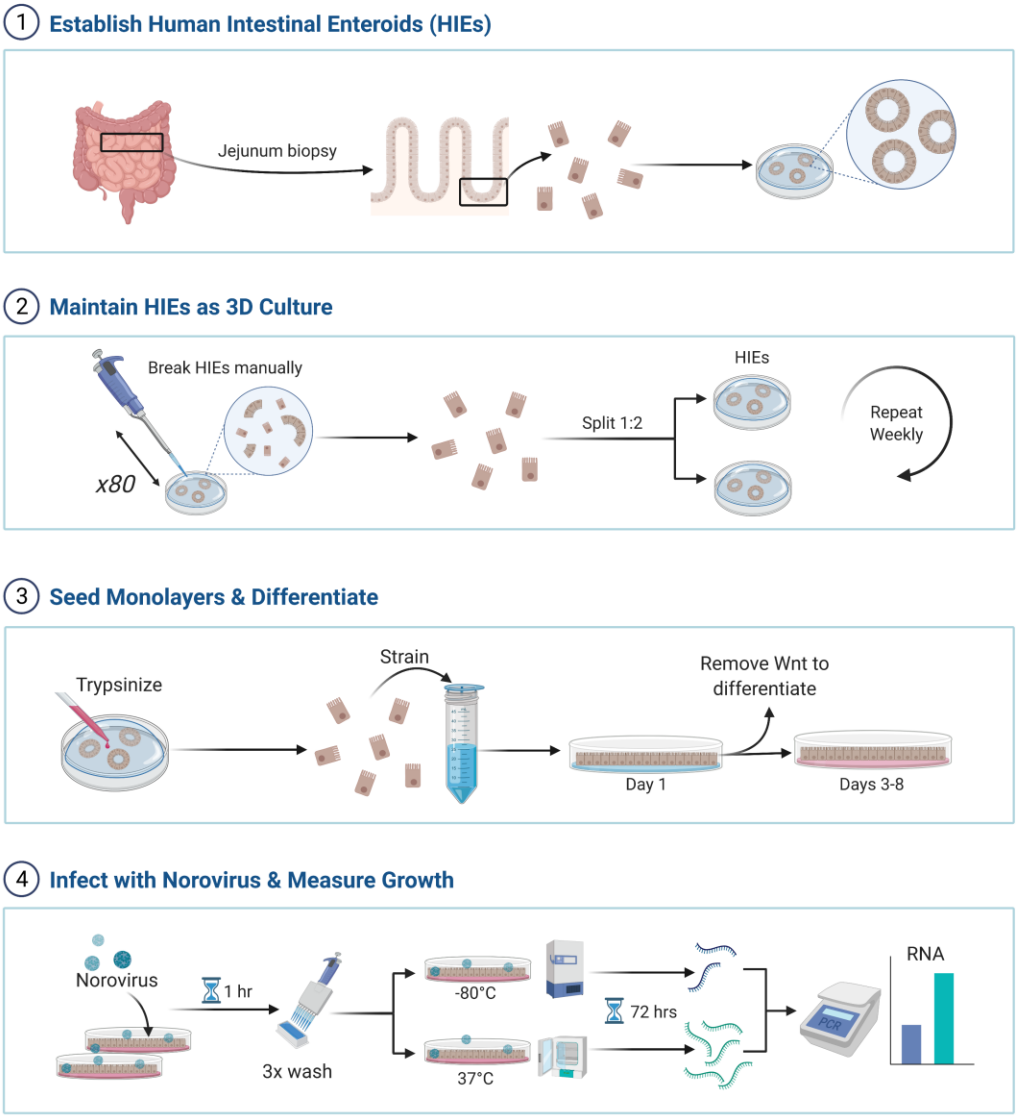
Table 2.3 Recommendations for codifying different types of experimental variables for studies that use human intestinal enteroids (HIEs) to grow human norovirus (HuNoV)

Experimental Variables	Primary Recommendations	Secondary Recommendations
HIE Cell Culture		
– Age of HIE cells	– Report HIE age as days of continuous growth between date of pulling HIE line from archive in LiN_2 and date of infection with HuNoV	– Record age of HIE information as passage number, days of continuous growth, and days archived – Examine the relationship between each age measure and resulting outcomes
– Basement membrane compound (BMC) used to seed HIE monolayers	– State BMC used and keep approach consistent throughout research project	– The use of Matrigel for growth is comparable or better than Collagen IV and cost per sample is lower
HuNoV Stool Suspension		
– Stool processing method	– 0.22 or 0.45 µm filtered 10% stool suspension is appropriate for non-disinfection applications of HIEs – If organic demand needs to be minimized (e.g. evaluating chemical disinfection), sucrose	– If higher HuNoV titer is required, the use of Vertrel XF or sucrose cushion ultracentrifugation should be considered

	cushion ultracentrifugation should be used	
– Infection volume added to HIEs	– For general application, 100µL inoculation volume is appropriate – For environmental monitoring applications where HuNoV titer may be low, a volume of 250µL is advisable	– Run replicates of the same samples with different volumes to compare HuNoV replication
Data Processing		
– Replacement method for below LOD values	– Clearly state the assay LOD and indicate the number of samples that fell below this value – Do not drop data below the LOD, replace with either half the LOD or the LOD	– Examine the effects that different LOD replacement values have on final data interpretation
– Choice of GE fold cutoff for identifying samples with infectious HuNoV	– State the fold cutoff used to determine if samples were positive for infectious HuNoV – Clearly identify when only positive samples are included in tables and figures	– Analyze data with multiple fold cutoff values to identify impacts on final interpretation

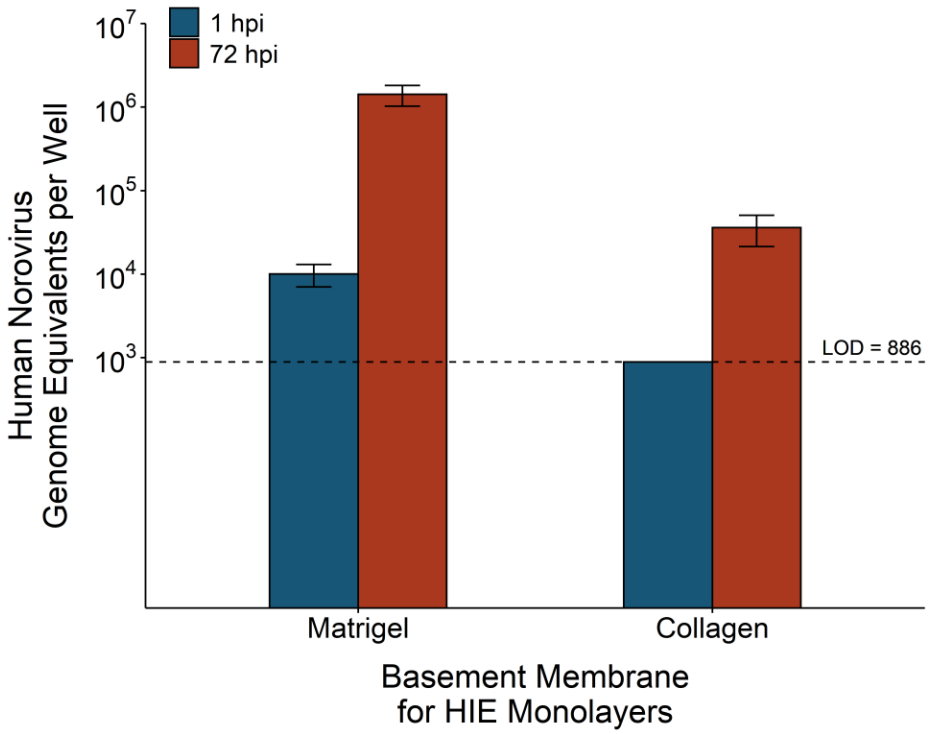
Figures

Figure 2.1 Method for growing human norovirus in human intestinal enteroids



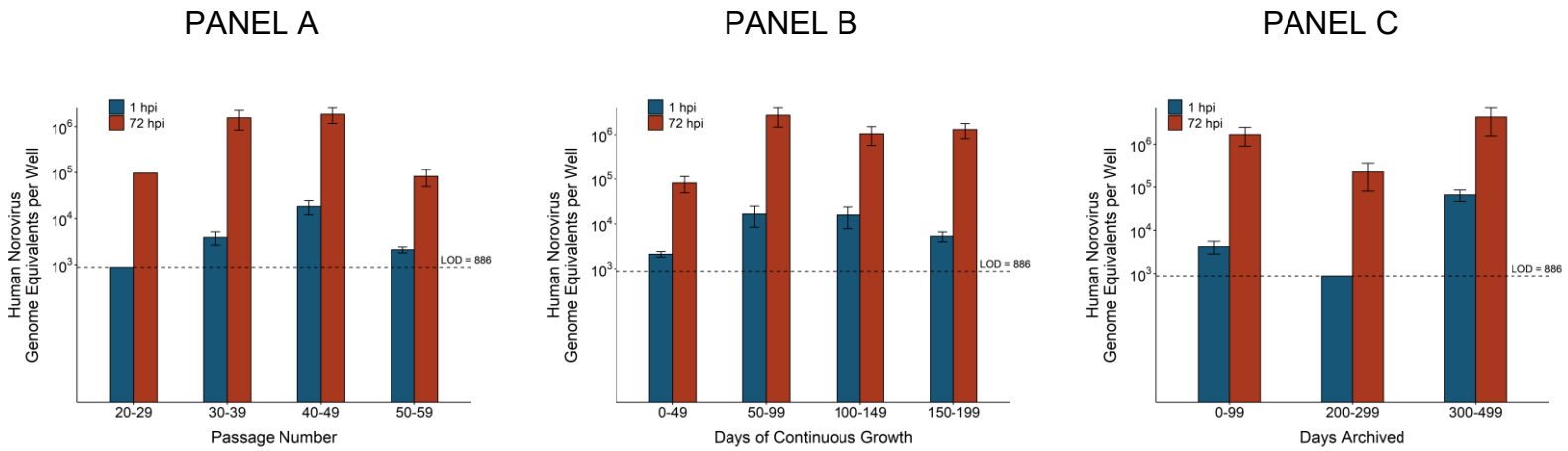
Created with BioRender

Figure 2.2 Human norovirus growth in human intestinal enteroids for two virus strains



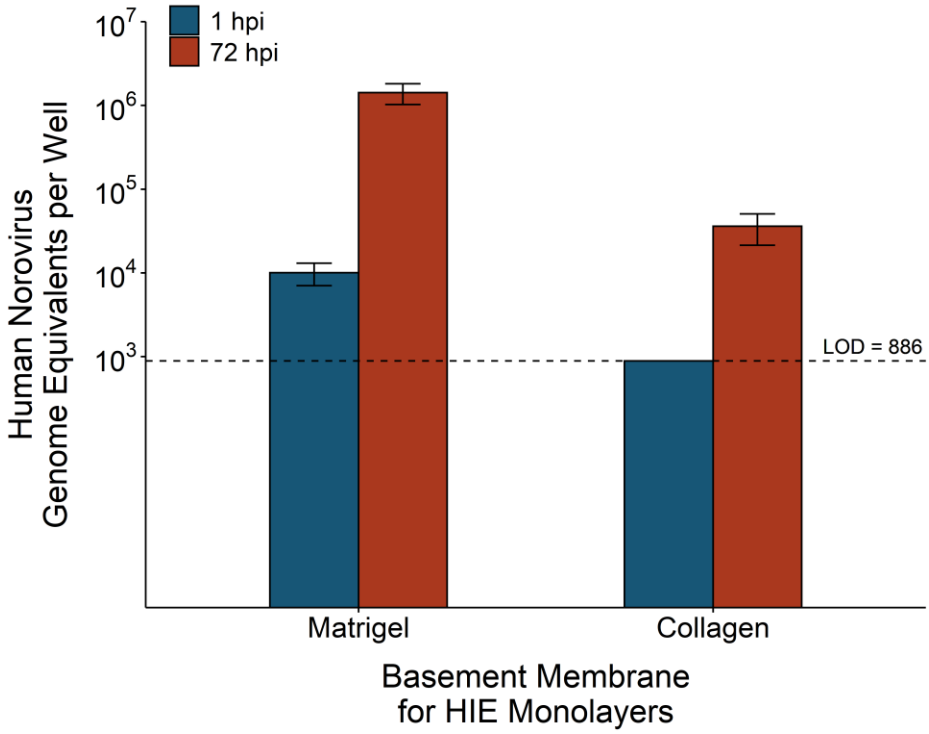
Human norovirus (HuNoV) growth as measured by increase in HuNoV genome equivalents (GE) between 1 and 72 hours post infection (hpi) in human intestinal enteroids (HIEs) for two virus strains: GII.4 Sydney (N = 18) and GII.4-16 Recombinant (N = 10). Data only includes experiments with positive detection of infectious HuNoV (fold change ≥5) and dashed line indicates assay limit of detection (LOD); error bars indicate standard error of the mean.

Figure 2.3 Effect of human intestinal enteroid age on human norovirus growth using different growth measures



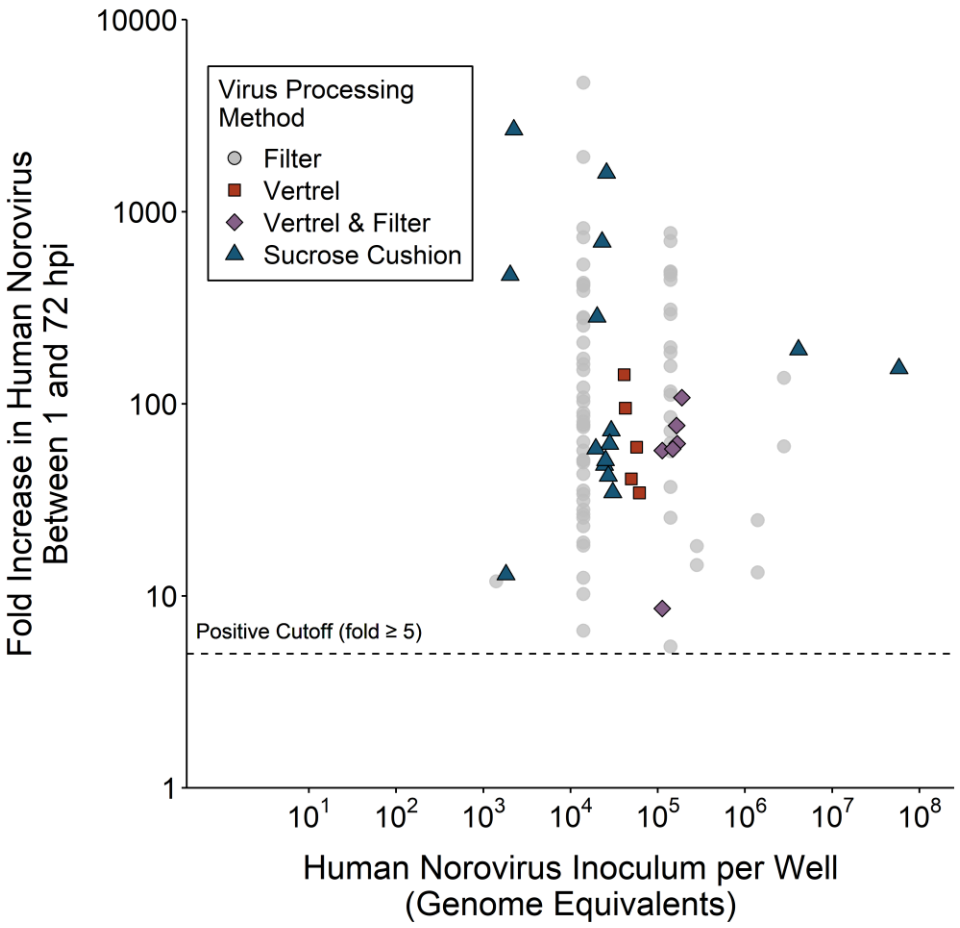
Human norovirus (HuNoV) growth as measured by increase in HuNoV genome equivalents (GE) between 1 and 72 hours post infection (hpi) in different ages of human intestinal enteroids (HIEs). Data only include experiments with positive detection of infectious HuNoV (fold change ≥5) and the dashed line indicates assay limit of detection (LOD); error bars indicate standard error of the mean. Panel A represents HIE age as passage number ranging from 20-29 (N = 1), 30-39 (N = 23), 40-49 (N = 35), and 50-59 (N = 6). Panel B represents HIE as days of continuous growth ranging from 0-49 (N = 6), 50-99 (N = 21), 100-149 (N = 17), and 150-199 (N = 20). Panel C represents HIE as the number of days cells were archived in liquid nitrogen and ranged from 0-99 (N = 21), 200-299 (N = 2), and 300-399 (N = 8)

Figure 2.4 Human norovirus growth in human intestinal enteroid monolayers seeded with two different basement membrane compounds



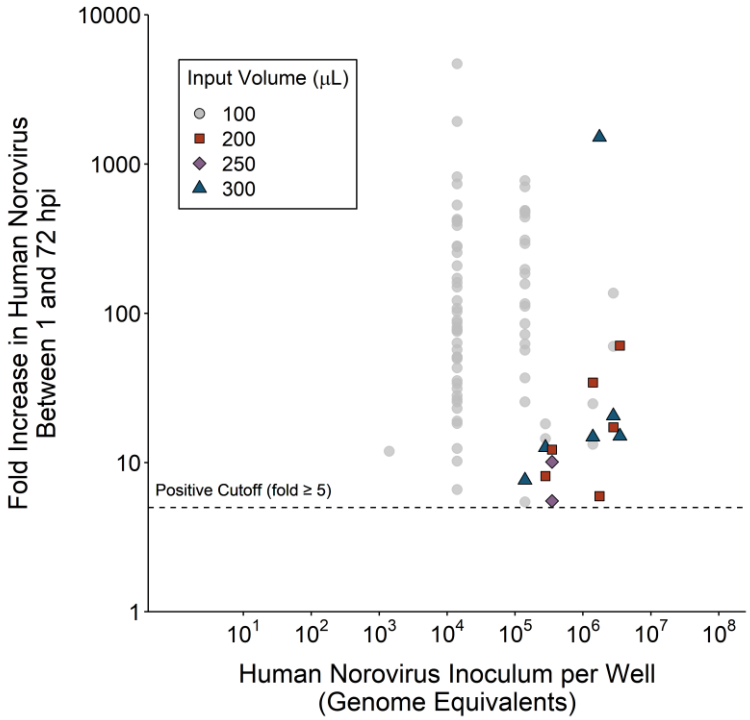
Human norovirus (HuNoV) growth as measured by increase in HuNoV genome equivalents (GE) between 1 and 72 hours post infection (hpi) in human intestinal enteroid (HIEs) monolayers for two basement membrane compounds: Corning Matrigel (N = 75) and Collagen IV (N = 7). Data only include experiments with positive detection of infectious HuNoV (fold change ≥5) and dashed line indicates assay limit of detection (LOD); error bars indicate standard error of the mean.

Figure 2.5 Relationship between initial dose of human norovirus and resulting growth in human intestinal enteroids for four viral inoculum processing methods



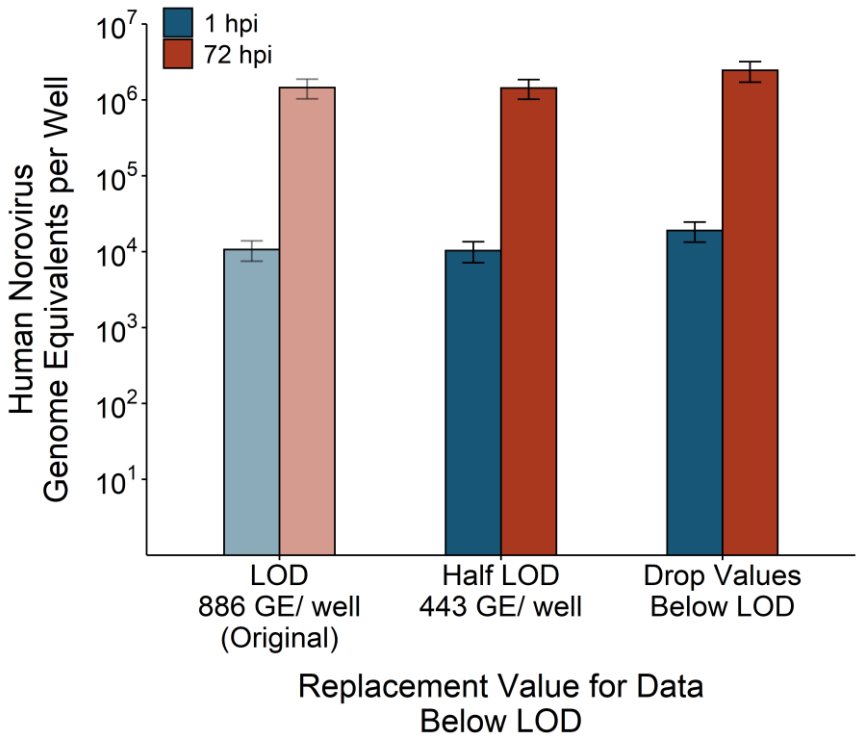
Inoculum of human norovirus (HuNoV) added to human intestinal enteroids (HIEs) and resulting HuNoV growth as measured by fold increase in HuNoV genome equivalents (GE) between 1 and 72 hours post infection (hpi) for four viral inoculum processing methods. Grey points indicate baseline experiments. Data only include experiments with positive detection of infectious HuNoV (fold change ≥5); assay limit of detection was 886 GE/ well.

Figure 2.6 Relationship between initial dose of human norovirus and resulting growth in human intestinal enteroids for four input volumes of viral inoculum



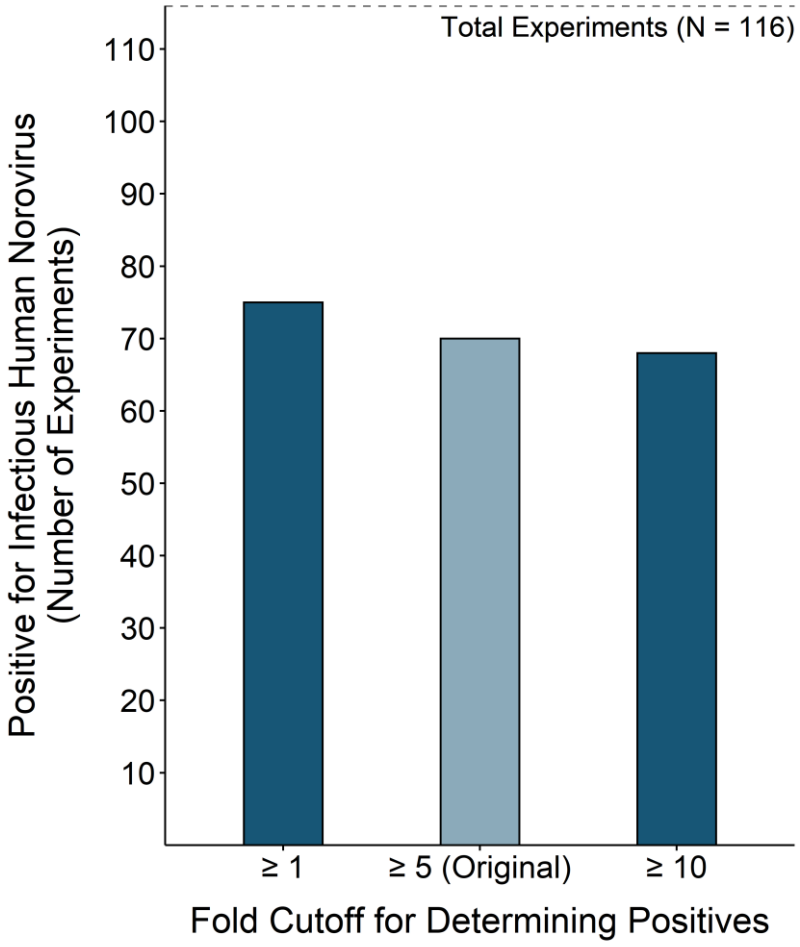
Inoculum of human norovirus (HuNoV) added to human intestinal enteroids (HIEs) and resulting HuNoV growth as measured by fold increase in HuNoV genome equivalents (GE) between 1 and 72 hours post infection (hpi) for four volumes of viral inoculum. Grey points indicate baseline experiments. Data only include experiments with positive detection of infectious HuNoV (fold change ≥5); assay limit of detection was 886 GE/ well.

Figure 2.7 Effect of value used to replace data below the limit of detection for human norovirus growth in human intestinal enteroids



Human norovirus (HuNoV) growth as measured by increase in HuNoV genome equivalents (GE) between 1 and 72 hours post infection (hpi) in human intestinal enteroid (HIEs) monolayers for three methods of replacing below limit of detection (LOD) values – replacement with the LOD value (lightened bar denotes baseline experiments, N=70), replacement with half of the LOD (N=71), and dropping values below the LOD (N=38). Data only include experiments with positive detection of infectious HuNoV (fold change ≥5); error bars indicate standard error of the mean.

Figure 2.8 Effect of varying the fold cutoff of HuNoV genome equivalents between 1 and 72 hours post infection for identifying human intestinal enteroid experiments that were positive for infectious human norovirus



Number of experiments that were considered positive for infectious human norovirus (HuNoV) in human intestinal enteroids (HIEs) for three different fold cutoffs -≥1, ≥5 (lightened bar denotes baseline experiments), and ≥10. Proportion of experiments positive for infectious HuNoV was not significant across the three groups (p=0.3). Below LOD values were replaced with the LOD of 886 genome equivalents/ well.

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CHAPTER THREE:

Recovery of infectious HuNoVs from fomites via replication in intestinal enteroids

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Abstract

Contamination of fomites by human norovirus (HuNoV) can initiate and prolong outbreaks. Fomite swabbing is necessary to predict HuNoV exposure and target interventions. Historically, swab-recovered HuNoV is measured by molecular methods that detect RNA but not infectious HuNoV. The recent development of HuNoV cultivation in human intestinal enteroids (HIEs) now enables detection of infectious HuNoV. It is unknown if the swabbing process and swab matrix will allow for cultivation of fomite-recovered HuNoV. We used HIEs to culture swab-recovered HuNoV from experimentally infected surfaces – a hospital bed tray (N=32), door handle (N=10), and sanitizer dispenser (N=11). Each surface was swabbed with macrofoam swabs premoistened in PBS plus 0.02% Tween80. Swab eluate was tested for infectious HuNoV by cultivation in HIE monolayers. Infectious HuNoV can be recovered from surfaces inoculated with at least 10^5 HuNoV genome equivalents/ $3cm^2$. In total, 57% (N=53) of recovered swabs contained infectious HuNoV detected by HIEs. No difference in percent positive swabs was observed between the three surfaces at p=0.2. We demonstrate that

fomite swabbing can be combined with the HIE method to cultivate infectious HuNoV from the environment filling a significant gap in HuNoV detection. Identification of infectious HuNoVs from swabs can increase monitoring accuracy, enhance risk estimates, and help prevent outbreaks. Currently, high titers of HuNoV are required to measure HuNoV growth in HIEs and the HIE system precludes absolute quantification of infectious viruses. However, the HIE system is capable of providing a binary indication of infectious HuNoV which enhances existing detection methods.

1 Introduction

Contamination of fomites by human norovirus (HuNoV) is a significant public health threat that can initiate and prolong outbreaks (1-4). Swabbing of fomites is an important approach to elucidating exposure patterns (5-9). Historically HuNoVs recovered from fomites have been detected by recovery of viral RNA with subsequent detection by reverse transcription-quantitative PCR (RT-qPCR). The advent of novel HuNoV culture methods offer new ways to fill important HuNoV knowledge gaps (10).

HuNoVs are the leading cause of acute gastroenteritis globally and cause significant health and economic burdens (11). Approximately 200,000 people will die of HuNoV every year and HuNoV infections cost the global economy $64.5

billion annually (12,13). HuNoV can be transmitted in a wide range of settings, including healthcare facilities, schools, and food service facilities (14-17)

HuNoV is spread through the fecal-oral route and virus transmission occurs from person-to-person contact, through aerosolized droplets, and from contact with contaminated fomites. In many community settings, fomite-based transmission is of particular concern due to long environmental stability of virus particles and low viral doses required for infection (18). There is evidence that fomites can initiate HuNoV outbreaks as well as lead to longer, more severe outbreaks (1-4).

In light of the important role of fomites in HuNoV transmission, numerous efforts have been undertaken to isolate and quantify HuNoV on environmental fomites. Swabbing is required to recover HuNoV from fomites and is used extensively in HuNoV outbreak investigations (19,20). Additionally, fomite swabbing is used to identify environmental HuNoV contamination outside of outbreaks as a means to prevent transmission, monitor control efforts, and understand epidemiologic trends (5-9). Swabs collected from the environment also contribute significantly to the knowledge base necessary to conduct HuNoV risk assessments (21-23). Fomite swabbing is also an important laboratory technique for identifying efficacy of cleaning and disinfection protocols for HuNoV (24).

Swabbing is important to HuNoV monitoring and research, but it remains

imperfect, with previous reports of low and inconsistent recovery of both HuNoV and other pathogens of human health significance (25) (26-28,9,23). A number of studies have also aimed to identify the most effective methods for swab recovery of HuNoV. Though no method is 100% effective, polyurethane foam swabs pre-moistened in PBS with Tween80 appear to have relatively consistent success in recovering HuNoV (29,28,25). The inconsistency in swabbing literature is due to the complexity required in development of a swabbing protocol. Researchers must choose swab material, buffer composition, surface type, recovery method, and detection method in order to balance efficient viral recovery with logistical considerations such as short sampling time (25).

Once HuNoV is recovered from swabs, the most common detection method is identification of HuNoV RNA by RT-qPCR. Molecular detection of HuNoV is necessitated by the historical inability to culture HuNoV in any known cell models (30,10). Molecular methods remain popular due to their high sensitivity, ease of use, and ability to provide robust quantification (31,32). However, molecular methods are unable to distinguish infectious HuNoV particles from inactivated RNA (32). The absence of clear data on HuNoV particle infectivity hampers risk assessments, environmental monitoring, and laboratory studies of disinfection.

One method to address the lack of a readily available HuNoV cell culture model

is the use of surrogate viruses. A wide range of surrogate viruses for HuNoV have been investigated, including non-human mammalian viruses and bacteriophages (33,34). The male-specific coliphage MS2 is one of the more commonly used HuNoV surrogates due to low cost, high replication in lab settings, absence of animal pathogenicity, similarity in size and genome to HuNoV, and ease of quantification in an *E. coli* plaque assay (35). MS2 has served as a valuable tool for laboratory studies of HuNoV fomite recovery and disinfection (36-39). However, no surrogate is perfect, and MS2 is unlikely to accurately model HuNoV disinfection (40-43). Additionally, surrogates cannot fill the gap in knowledge around prevalence of infectious HuNoV that is required for robust risk assessments.

The newly developed human intestinal enteroid (HIE) model for cultivation of HuNoV offers promise in filling the gaps left by molecular detection and surrogate studies (10). The HIE approach, introduced in 2016, represents the first successful attempt to culture HuNoV (10). Multiple researchers have demonstrated the reproducibility of HuNoV replication in monolayers seeded from stem-cell derived HIEs (44-46,10,47). The HIE method relies on measuring fold increase in viral RNA between 1 and 72 hours post infection which precludes absolute quantification of viral particles (48). Due to the nature of HIE cells, no direct quantification, like that achieved with plaque assay, can occur (10). Previous work has also indicated a wide variability in HuNoV replication even

with consistent inputs (46). Additionally, the HIE cell model is resource and time intense, requiring multiple weeks of growth in order to process a single sample (48). Despite these challenges, HIEs remain the only way to cultivate HuNoV and offer the opportunity to address gaps in our understanding of HuNoV prevalence, risk modeling, and susceptibility to disinfectants.

Use of the HIE model to cultivate swab recovered HuNoV is necessary to measure population exposures, target areas for intervention, enhance risk assessment data, and conduct disinfection studies. However, the HIE method has not been applied to cultivation of swab-recovered HuNoVs. We investigated how the complex swab matrix, which often includes salts and surfactants, impacts HuNoV replication in HIE cells. Additionally, we determined if the process of swabbing and recovery will yield in-tact HuNoV that is capable of replication in HIE cells.

2 Methods

2.1 Viral Stock Preparation

HuNoV stool suspensions were prepared from a community pediatric HuNoV case that was graciously provided by Dr. Natalie Exum. Stool was lab confirmed for HuNoV by RT-qPCR and identified as a GII.4 Sydney virus based on the

capsid region (49). Raw stool was diluted to 10% in phosphate buffered saline (PBS) and filtered through a 0.45μm filter. Samples were portioned and stored at -80°C from collection until time of testing. MS2 stocks were propagated and purified with ultra-membrane filtration before portioning and storage at -80°C, as previously described (35,41).

2.2 Fomite Preparation and Inoculation

Three items representing common high touch fomites found in community settings were tested for HuNoV recovery in this study - a hospital bed tray (melamine-laminate), a lever-style door handle (brushed stainless steel), and a hand sanitizer dispenser (acrylonitrile butadiene styrene (ABS) plastic). All items were kindly provided by Johns Hopkins Hospital Facilities Management. Each item was marked with multiple 3 cm^2 areas for sampling; swab areas were on the top surface of the bed tray, the smooth grab surface of the door handle, and the front of the push lever of the sanitizer dispenser (Figure 1). Prior to inoculation, fomites were disinfected with sequential applications of 10% bleach, 70% ethanol, and distilled water. Each fomite was then exposed to a UV lamp (253.5nm) for 30 minutes.

Fomite inoculum consisted of a 10% dilution of HuNoV stool suspension in PBS. MS2 diluted in PBS, was added to a subset of HuNoV fomite inoculums. Unless

stated otherwise, 50µL of inoculum was applied to the fomite per swabbing experiment. After inoculation, each fomite was immediately swabbed horizontally, vertically, and then diagonally (Figure 2).

2.3 Swabbing Method

Individually wrapped, sterile 100% polyurethane foam (PUF) swabs (STX708A, Texwipe, Kernersville, NC, USA), common in industry fomite monitoring (29), were used to recover HuNoV from inoculated fomites. Prior to swabbing, each swab was placed in a 15mL conical tube that contained 2mL PBS plus 0.02% Tween80 detergent (Millipore Sigma, Burlington, MA, USA). Tween80 was added to swabbing medium as it has been shown to increase recovery of microorganisms from fomites (28). Swab medium was weighed before and after swabbing to measure volume loss and calculate final eluate volume. To recover viruses, swab-containing tubes were vortexed for 30s, centrifuged for 1 min, and then liquid was manually recovered from foam swab heads by pressing the swab stick along the side of the tube until no further liquid could be squeezed out. After elution, recovered swab medium was stored at -80C until testing.

2.4 RNA Extraction and Detection via RT-qPCR

Total RNA was extracted from swab eluate using Ribozol (VWR, Radnor, PA,

USA) and the Direct-Zol RNA purification kit (Zymo Research, Irvine, CA, USA) as described previously (10). RNA was detected and quantified using the QuantiTect Probe RT-PCR Kit (Qiagen, Hilden, Germany). Primers and probes specific to the MS2 replicase gene (35) or the HuNoV ORF1-ORF2 junction (50) were used in the RT-qPCR assay. MS2 was quantified from molecular assays with an RNA dilution series with known amounts of coliphage. HuNoV RNA was converted from a cycle threshold value (Ct) to genome equivalents (GE) using in vitro RNA transcripts kindly provided by Michael Kulka (FDA, Silver Spring, MD, USA). Transcripts were derived from plasmid pNoV/MD145 which contained a full-length synthetic cDNA copy of a HuNoV GII strain (51). Molecular percent recovery was calculated by comparing HuNoV GE applied to surface to HuNoV GE in total recovered swab eluate. We confirmed the absence of RT-qPCR inhibition for HuNoV and MS2 assays with a spiked internal positive control (data not shown).

2.5 Infectivity Methods

The HIE method for culturing HuNoV has been described in detail previously (46,10). Briefly, a secretor-positive jejunal HIE cell line (J2), kindly provided by Mary Estes (Baylor College of Medicine, Houston, TX), was maintained as undifferentiated three-dimensional (3D) (i.e., spheroid) cultures embedded in Matrigel (Corning, Corning, NY, USA). HIEs were maintained at 37°C in 5% CO_2

and Human IntestiCult media (STEMCELL Technologies, Vancouver, Canada). After 7 days of growth, 3D cultures were either passaged 1:2, archived in LiN2, or used to seed monolayers. Passaged HIE monolayers were grown for two days in IntestiCult supplemented with 10 μM Y-27632 (ROCK Inhibitor), 10 μM CHIR99021 (GSK3 inhibitor) (STEMCELL Technologies, Vancouver, Canada) and 1000μm / mL Primocin antimicrobial agent (InvivoGen, San Diego, CA, USA). After two days CHIR99021 was removed from the growth media.

To seed monolayers, HIEs were dissociated to a single cell suspension with Trypsin and plated 1:2 as undifferentiated monolayers in Matrigel-coated 96-well cell culture plates. Monolayers were grown for two days with IntestiCult supplemented with 10 μM Y-27632 and then differentiated for 5 days prior to infection with media lacking Wnt3a, R-spondin-1, and SB202190 (p38 MAPK inhibitor), as previously described (52,53).

Confluent, differentiated HIE monolayers were infected apically in duplicate and all infection media was supplemented with 500 μM of glycochenodeoxycholic acid (GCDCA; Sigma-Aldrich, St. Louis, MO, USA). After 1 hour of incubation at 37°C in 5% CO_2, supernatant was removed and monolayers were washed three times with complete media without growth factors. For each set of infections, one monolayer was immediately frozen at -80°C and the second was grown at 37°C

in 5% CO_2 for 72 hours post infection (hpi). Following the 72-hour incubation, the supernatant and monolayer cells were frozen at -80°C. We then extracted RNA from 1 hpi and 72 hpi monolayer cells and supernatants.

A standard 10-fold dilution, double agar plaque assay was used to enumerate infectious MS2 coliphage as plaque forming units (PFU) following the protocol described by Bae and Schwab (35).

2.6 Statistical Methods

Statistical analyses were performed in Stata 13 and R 3.6.1 (54,55). HuNoV replication was measured as the fold increase between HuNoV RNA copies measured at 1 and 72 hpi; samples were considered negative for replication if the fold increase was less than five. Values below the RT-qPCR LOD (44.3 RNA copies/ 5uL) were replaced with the LOD value.

3 Results

Fifty individual swabbing experiments were performed: 29 on a hospital bed tray, 10 on a lever-style door handle, and 11 on a hand sanitizer dispenser. Of these 50 experiments, 30 were positive for infectious HuNoV as measured by a 5-fold or greater increase in HuNoV RNA copies between 1 and 72 hpi in HIEs. Based

on 14 runs of seven dilutions in duplicate, the RT-qPCR limit of detection (LOD) was determined to be 44.3 viral RNA copies/ 5uL, as calculated using the discrete threshold method (56).

Bed tray experiments were used to identify the volume and range of HuNoV inoculum required for successful replication. Ten swabs were recovered from the bed tray after inoculation with 100µL of HuNoV stool suspension ranging in concentration from 2.8 x 10^5 to 2.8 x 10^6 HuNoV GE/ cm^2 (Figure 3). Three (30%) of the 100µL inoculum fomite-recovered swabs were positive for infectious HuNoV. Additionally, 50µL inoculum was used in 19 bed tray swab experiments with HuNoV concentrations from 3.5 x 10^4 to 8.7 x 10^6 HuNoV GE/ cm^2 (Figure 3). Eleven (58%) of the 50µL inoculum fomite-recovered swabs were positive for infectious HuNoV. The lowest fomite inoculum that resulted in recovery and detection of infectious HuNoV was 1.4 x 10^5 GE/ cm^2. Less than 50% of swabs from fomites inoculated in the 10^5 HuNoV GE/ cm^2 contained measurable infectious HuNoV, even with similar amounts of viral input (Figure 3). Viral inoculum of 10^6 HuNoV GE/cm^2 or greater resulted in 0% recovery when 100µL fomite inoculum was used and 100% recovery when 50µL inoculum was used.

Once it was established that 50µL HuNoV stool suspension with at least 1.4 x 10^5 HuNoV GE/ cm^2 led to successful recovery of infectious HuNoV from fomites,

we measured recovery from metal door handles and plastic sanitizer dispensers inoculated with 10^6 or greater HuNoV GE/ cm^2. The percent of swabs that were positive for infectious HuNoV from bed tray, door handle, and sanitizer dispenser experiments were 100% (n = 7), 80% (n = 10), and 73% (n =11), respectively. Door handle-recovered swabs had the highest HuNoV replication, as measured by fold increase in HuNoV GE between 1 and 72 hpi in HIEs. The average fold increase in HuNoV GE was 2.3 x 10^3 (SD 3.1x10^3, n = 10) for door handle experiments, 1.1 x 10^3 (SD 1.3 x 10^3, n = 11) for sanitizer dispenser experiments, and 2.7 x 10^2 (SD 3.3 x 10^2, n = 7) for bed tray experiments (Figure 4). Recovery of infectious HuNoV was not significantly different across the three fomites when considering percent of positive swabs (ANOVA p-value = 0.5) or when considering measured fold increase (ANOVA p-value = 0.2).

Swabs recovered from fomites infected with 50µL of HuNoV stool suspension were tested for molecular HuNoV recovery, in addition to HuNoV replication. Average percent recovery of HuNoV measured by molecular methods was 0.74% (range 0.03% to 4.3%) and was not significantly different across the three tested fomite types (ANOVA p-value = 0.3) (Figure 5). Twelve of 40 swabs had molecular HuNoV recovery below 0.1% and 8 of these swabs were positive for infectious HuNoV. Percent of swabs positive for infectious HuNoV in the two higher categories of molecular recovery - 0.1 to 1% and above 1% - were 77% (7 of 9) and 71% (5 of 7), respectively (Figure 5). Twelve swabs were negative for

HuNoV by molecular methods; five of these were also negative for infectious HuNoV. When controlling for HuNoV GE on fomite and fomite type, no relationship was found between detection of infectious HuNoV and molecular HuNoV percent recovery (binomial regression, all p-values > 0.04).

In addition to HuNoV stool filtrate, fomite inoculum for 25 swabbing experiments contained MS2 ranging from $1.86 \times 10^2 – 3 \times 10^6$ GE/ cm^2. Infectious MS2 measured by plaque assay was found in all swab experiments run with MS2, and MS2 RNA was also detected in 18 of these swabs (Figure 6). Two bed tray-recovered swabs negative for MS2 RNA but not infectious MS2 were from fomites inoculated with $< 2 \times 10^3$ MS2 GE/ cm^2. The other 7 swabs negative for MS2 RNA and positive for infectious MS2 were from door handle or sanitizer dispenser experiments with 3.1×10^4 MS2 GE/ cm^2 in fomite inoculum. Average percent recovery of MS2 RNA was 74% by RT-qPCR and average percent recovery of infectious MS2 was 75% by plaque assay; no difference in either measure was found across fomites types (ANOVA p-values = 0.9 and 0.4).

4 Discussion

Fomite transmission of HuNoV plays an important role in initiating and prolonging outbreaks. Additionally, the presence of HuNoV on fomites can inform measures of public health risk, identify targets for intervention, and indicate the efficacy of

inactivation methods. Fomite swabbing is necessary to understanding the distribution, exposure, and persistence of HuNoV. The pioneering development of an HIE model for culturing infectious HuNoV promises to fill in important gaps around detection of infectious HuNoV particles after recovery from fomites (10). However, the HIE model faces numerous logistical hurdles before it can be readily applied in fomite recovery research (48). This work aimed to tackle the first of these hurdles - whether swab recovered virus can successfully replicate in HIEs. We demonstrated that HuNoV can be experimentally applied to fomites, recovered via swab, eluted, and subsequently replicate in HIE cell culture. Swab eluate comprised of PBS plus Tween80 does not prevent growth of HIEs or replication of HuNoV in HIE culture, paving the way for use of the HIE model to cultivate swab-recovered HuNoV. Our base experiments used a hospital bed tray with a textured, laminated surface, as this type of fomite is common in health care settings where fomite swabbing is particularly necessary (8). We determined that at least 1.4×10^5 HuNoV GE/ cm^2 must be present on a surface in order to successfully recover infectious HuNoV. Accounting for losses from swabbing, this value is consistent with previous reports of 10^3 - 10^4 GE/ HIE well as a requirement for successful HuNoV replication (46,10). We also found that high viral titer applied to the surface in larger volumes (100µL) did not result in successful recovery of infectious HuNoV. This may be due to dilution introduced by larger volumes and the inability of the pre-moistened swab to fully recover large inoculum volumes. Importantly, HuNoVs are frequently shed at high titers

and thus small droplets inoculated onto fomites can be clinically relevant (57).

We first measured presence of infectious HuNoV as binary, where a swab was considered positive if we observed a 5-fold or greater increase in GE between 1 and 72 hpi in HIEs. The number of swabs positive for infectious HuNoV was inconsistent even within equivalent surface inoculum categories, except for high viral titer (>10^6) in 50µL of inoculum. This indicates that high concentration viral titers provide the most successful and consistent recovery of positive virus, consistent with previous work on molecular recovery of HuNoV (58). These values can guide future bench-scale evaluations of HuNoV fomite inactivation and having the HIE system used as a binary measure of infectious HuNoV post-fomite disinfection will be a powerful first step when developing risk models. Quantification may be possible by inoculating portions of samples in a dilution series into the HIE system, with subsequent enumeration of viral load using the most probable number (MPN) method. Another potential option for quantifying recovered infectious HuNoV is the fold increase in GE between 1 and 72 hpi in HIEs. Consistent with previous work, we found that fold increases ranged from 10 - 10,000 and varied from 2 - 3 logs within tests that used equivalent surface inoculum (45,46,59). We are not the first to report high variability among measured fold increase in HIEs and this inconsistency remains a key challenge for application of the HIE system to monitoring infectious HuNoV (46,48,47).

In addition to the melamine-laminate bed tray, we tested two other fomites common in healthcare settings - a brushed stainless steel lever-type door handle and a smooth ABS plastic sanitizer dispenser. . We found no measurable differences in recovery of infectious HuNoV off of these two fomites, compared to bed tray experiments. This is promising for future environmental monitoring work as the data suggest that multiple types of fomites can be swabbed for infectious HuNoV.

We measured molecular recovery of HuNoV with RT-qPCR to serve as a point of comparison with infectious HuNoV data. We found that average recovery of HuNoV from fomites as measured by molecular methods was 0.74% and ranged from 0.03% to 4.3%. These recovery values are slightly lower than most reported in the literature, which range from 4.3% - 100% (26,60,27,61,62,58). However, it is important to note that previously reported recovery of HuNoV from hard surfaces is highly variable both within and across studies and when reported errors are accounted for, our observations fall within previously reported ranges (29,9,62,25). Of note, even when molecular recovery of HuNoV was below 0.1%, approximately half of swabs were positive for infectious HuNoV. It appears that even in scenarios with low molecular recovery, infectious HuNoV particles can still be collected from fomites. We found that a few swabs were negative for

HuNoV as measured by RT-qPCR, but were positive for infectious virus. This discrepancy between infectivity and molecular measures has been observed in other viruses and is likely due to methodological limitations of the RT-qPCR assay (63,64). Additionally, a smaller amount of sample (5µL) was tested in RT-qPCR runs, as compared to 250µL for HIE infection, which may reduce the efficacy of RT-qPCR for low-titer samples.

As is common in HuNoV literature, we also tested the surrogate virus MS2 coliphage in a subset of swabbing experiments with both molecular and infectivity methods (65,66). Recovery of MS2 via molecular (74%) and infectivity (75%) methods were comparable, which provides validity to our experimental set-up. However, MS2 recovery was 2 logs greater than molecular HuNoV recovery (0.74%). This recovery variation between viruses, particularly those used as surrogates, has been described in detail in the literature (67,62). Differences in virus structure can affect viral adhesion to fomites, containers, and swabs, which can then impact recovery (68). Our work adds to the extensive literature that questions the accuracy of MS2 coliphage as a HuNoV surrogate (35,41,69,70). Though MS2 retains value due to its ease of cultivation, the data from this study shows that it cannot be employed as a replacement for measuring HuNoV recovery from fomites. Thus, even when faced with multiple methodological hurdles, the HIE system has significant value as it remains the only option for specifically identifying infectious HuNoV.

This work is subject to limitations that were beyond the scope of the current project. First, we evaluated the behavior of a single HuNoV strain - GII.4 Sydney - as previous work on HIEs indicates that this strain replicates most successfully (46). The variation in replication of different HuNoV strains is a well-documented limitation of the current HIE method (46,48,10). Low sample sizes due to methodological limitations of the HIE method should also be considered when generalizing specific recovery measurements from this work. We also focused on a single swabbing protocol and though the literature indicates good success with foam swabs pre-moistened in PBS plus Tween80, we could be missing recovery variability found by other methods (61). Additionally, we did not examine drying as we were focused on confirming fomite recovery of infectious HuNoV and previous literature indicates a significant reduction in recovery after drying (61). Finally, we did not address the role that viral aggregation may play in observed recovery efficiencies (68).

We have successfully demonstrated that the HIE culture method can be used to cultivate infectious HuNoV recovered from fomites under prescribed conditions. This adds new utility to the HIE method and opens the door for numerous studies aimed at cultivating fomite recovered virus. Though the HIE method remains an imperfect tool, our work offers a blueprint for moving forward with fomite

monitoring and disinfection studies. The most important next steps will be to address some of the hurdles that prevent wide application of the HIE system in monitoring. It will be important that future work examines factors that impact inconsistent replication of HuNoV in HIEs and aims to develop reliable methods of quantification. Additionally, reduction of the time, labor, and expense required to use HIEs for HuNoV cultivation will significantly increase the applicability of the method. The HIE method remains the only widely reproducibly way to verify infectious HuNoV and our ability to recover and cultivate swab-recovered viruses moves the field one step closer to a broadly applicable system that can measure infectious HuNoV in the environment.

Acknowledgements

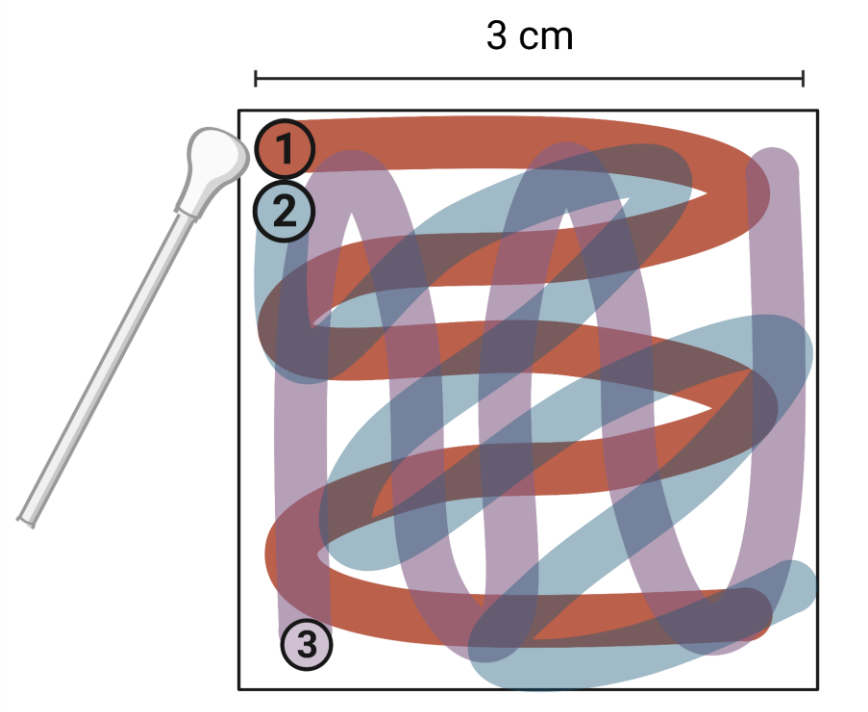
Authors would like to thank Samantha Wales from FDA CFSAN and Sunny Lee at Johns Hopkins School of Medicine for technical assistance in culturing HIEs. The authors also wish to acknowledge the Integrated Physiology Core of the Hopkins Conte Digestive Disease Basic and Translational Research Core Center for providing enteroid differentiation media.

Figures

Figure 3.1 Surfaces tested for human norovirus swab recovery.

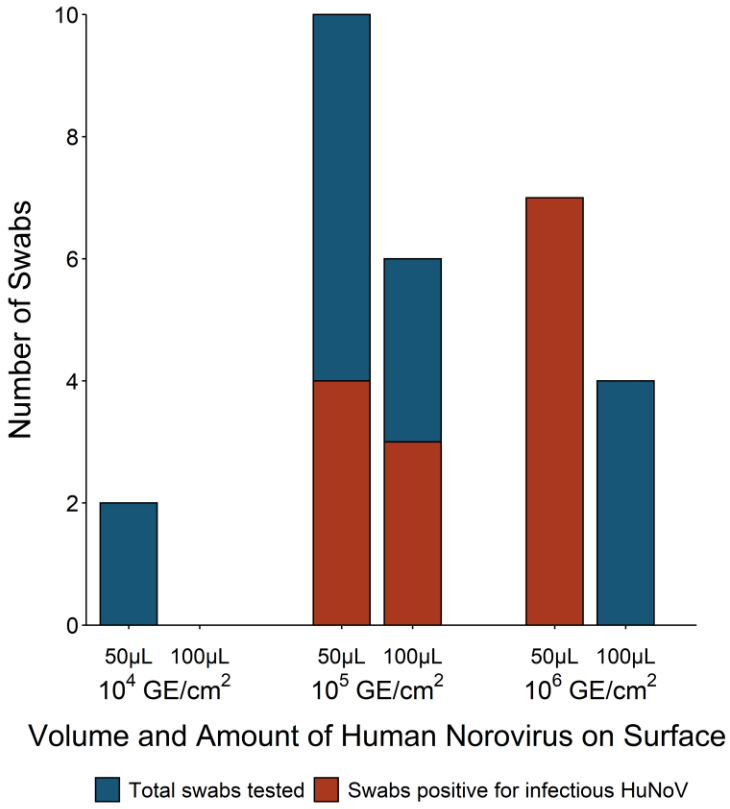
Red boxes indicate $3cm^3$ areas that were inoculated and swabbed.

Figure 3.2 Direction and order of swabbing on surfaces tested for human norovirus swab recovery.



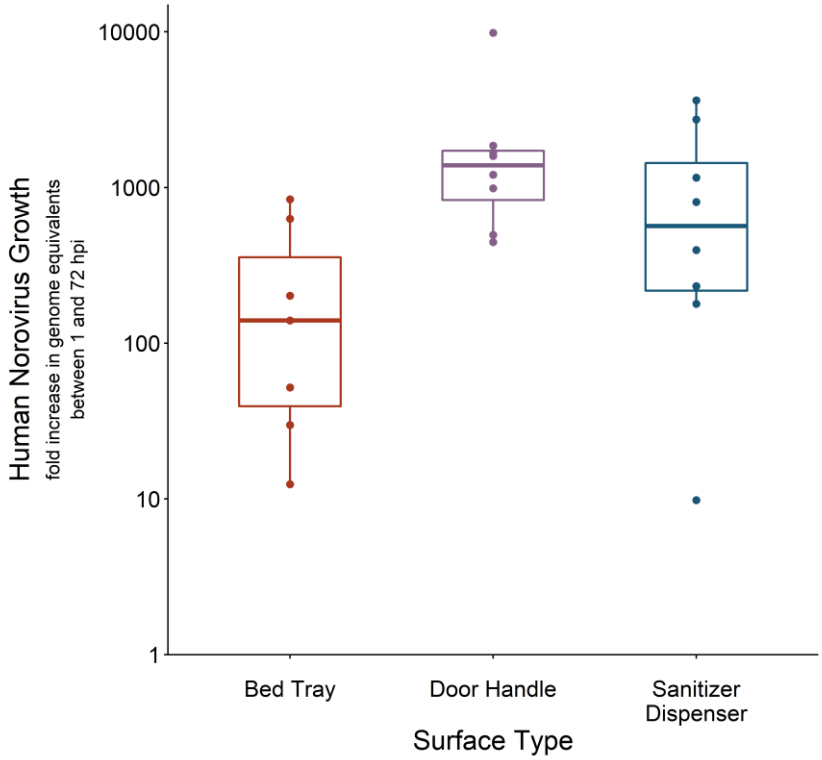
Created with BioRender

Figure 3.3 Number of fomite-recovered swabs that were tested and positive for infectious HuNoV (HuNoV) by surface inoculum volume and amount of HuNoV on fomite (genome equivalents(GE)/ cm2).



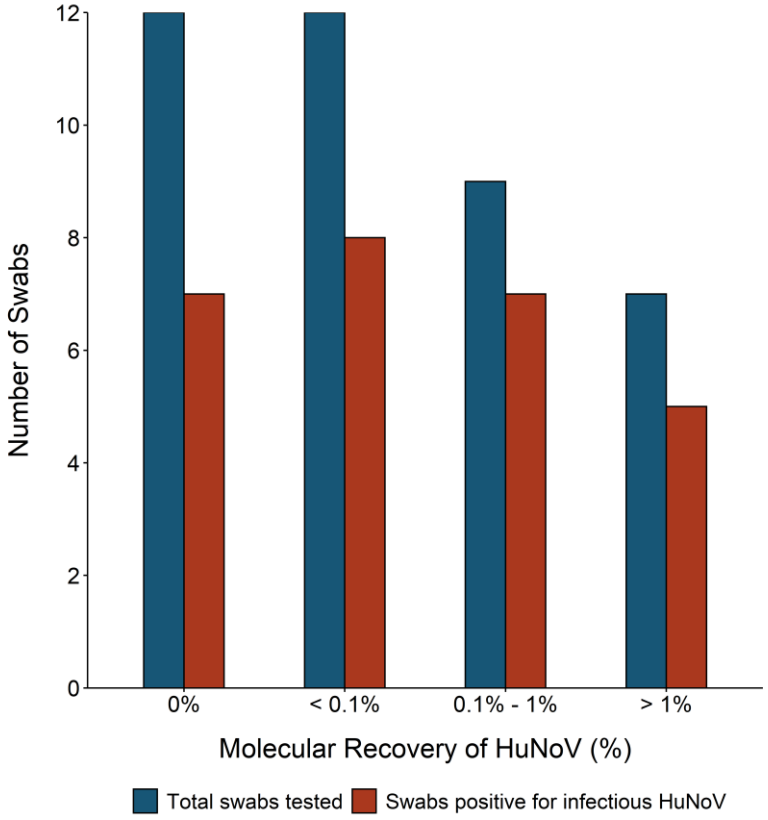
Non-porous laminated fiberboard from a hospital bed tray was inoculated with either 50 or 100µL HuNoV stool suspension containing 10^4, 10^5, or 10^6 HuNoV GE/ cm^2. Fomites were swabbed with macrofoam swabs pre-moistened in phosphate buffered saline plus 0.02% Tween80. Human intestinal enteroid (HIE) monolayers were infected with swab eluate and were considered positive for infectious HuNoV if the fold increase in HuNoV GE between 1 and 72 hours post infection (hpi) exceeded five.

Figure 3.4 HuNoV (HuNoV) replication from swabs recovered off lab-inoculated fomites.



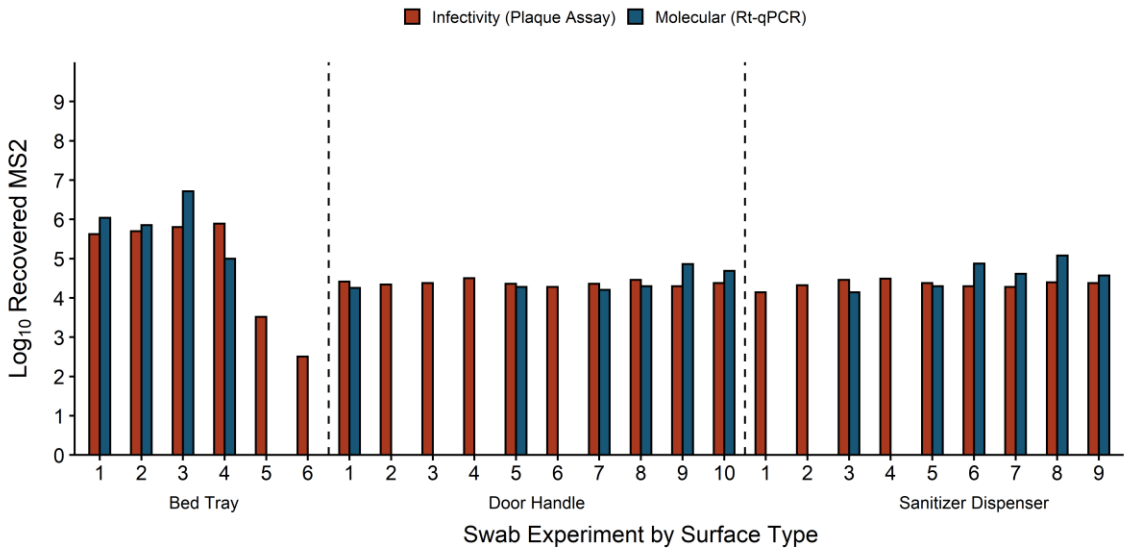
Three fomites - a laminated fiberboard hospital bed tray (n = 7), a brushed stainless steel door handle (n =8), and an acrylonitrile butadiene styrene (ABS) plastic sanitizer dispenser (n = 7) - were inoculated with 50µL HuNoV stool suspension containing 10^6 or greater HuNoV genome equivalents (GE) per cm2. Fomites were swabbed with macrofoam swabs pre-moistened in phosphate buffered saline plus 0.02% Tween80. Human intestinal enteroid (HIE) monolayers were infected with swab eluate and HuNoV replication is reported as the fold increase in HuNoV GE between 1 and 72 hours post infection (hpi). Fold-increase of five or lower was considered negative for infectious HuNoV; only swabs positive for infectious HuNoV are shown.

Figure 3.5 Number of swabs tested and with detectable infectious HuNoV (HuNoV) compared to molecular percent recovery of HuNoV from experimentally inoculated fomites.



One of three fomites - hospital bed tray (n = 19), door handle (n = 10), or sanitizer dispenser (n = 11) - were inoculated with 50µL of HuNoV stool suspension containing 10^4 or greater HuNoV genome equivalents (GE) per cm^2. Fomites were swabbed with macrofoam swabs pre-moistened in phosphate buffered saline plus 0.02% Tween80. Human intestinal enteroid (HIE) monolayers were infected with swab eluate and were considered positive for infectious HuNoV if the fold increase in HuNoV GE between 1 and 72 hours post infection (hpi) was greater than five. Molecular percent recovery of HuNoV was calculated by comparing HuNoV GE added to fomite to HuNoV GE in recovered eluate, using RT-qPCR.

Figure 3.6 Recovery of MS2 by infectivity and molecular methods for each individual swab experiment.



MS2 recovery is equivalent to plaque forming units for infectivity and genome equivalents (GE) for molecular. Fomites were inoculated with 50µL HuNoV stool suspension mixed with 10^2 - 10^7 MS2 GE/ cm^2. Fomites were swabbed with macrofoam swabs pre-moistened in phosphate buffered saline plus 0.02% Tween80. Swab eluate was tested for MS2 replication with an E. coli plaque assay and for MS2 molecular recovery using RT-qPCR.

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CHAPTER FOUR:

Quantitative microbial risk assessment of human norovirus infection in environmental service workers due to healthcare-associated fomites

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Abstract

Objective: To quantify human norovirus (HuNoV) infection risk in healthcare environmental service workers (ESWs) from a single fomite contact in areas occupied by HuNoV positive patients. An additional aim was to further quantify the risk of secondary HuNoV transmission and illness in healthcare settings as a result of fomite transfer from ESWs.

Design: A quantitative microbial risk assessment employing 2D Monte Carlo to model variability and uncertainty of parameters to estimate HuNoV infection in multiple fomite contact scenarios. Scenarios were defined by: source of HuNoV (vomit or diarrhea), location (bathroom or patient room), and target outcome (ESW illness or secondary illness).

Setting: Healthcare facilities in the United States

Results: The risk of an ESW becoming ill with HuNoV was as high as 1:3 for single fomite contact in a room where a patient experienced diarrhea. Risk estimates for vomit scenarios were four orders of magnitude lower than those for diarrhea scenarios. Risk of secondary illness from touching a common surface was 32% lower than risk of ESW illness in diarrhea scenarios and 80% lower in vomit scenarios.

Conclusions: A single fomite contact can lead to sizable HuNoV infection risk in ESWs, who can also transfer virus to secondary surfaces, initiating further infections. ESWs likely face occupational health risks from fomite-mediated infections and robust interventions are needed.

1 Introduction

Human noroviruses (HuNoVs) cause approximately 685 million illnesses every year and cost the global economy $64.5 billion annually (1-3). In many countries, including the United States, the majority of HuNoV outbreaks occur in healthcare facilities, including hospitals and long-term care facilities (4,5). The burden of healthcare-associated norovirus infections is high, and the presence of immunocompromised and elderly patients can prolong outbreaks in these settings, exposing health care providers for months at a time (6-12). In many healthcare-associated outbreaks, staff make up the majority of cases (8,9). HuNoV infections in healthcare facilities can lead to significant monetary losses

and reduction in hospital capacity due to closures and ill staff (13,14). For example, a single HuNoV outbreak in a tertiary care hospital in 2004 led to 265 healthcare worker illnesses and an estimated cost of $657,644 (9).

HuNoVs possess multiple characteristics that make them a significant pathogen in healthcare settings. HuNoVs are shed in high titer from the vomit and stool of symptomatic individuals (15,16). Of particular challenge, there is frequent asymptomatic shedding of infectious virus (17), and the infectious dose of HuNoVs is very low, estimated to be between 10-100 particles (18). In a healthcare setting, the presence of immunocompromised patients can increase the likelihood of HuNoV spread as these patients can chronically shed high titers of HuNoV (4,19).

While multiple studies have aimed to quantify the impact of HuNoVs of healthcare staff, a vast majority of these studies have focused on nurses, doctors, and other clinical staff (8,20,11,14). In many labor sectors, including healthcare, cleaning and facilities staff are frequently understudied (21,22). In the healthcare setting, environmental service workers (ESWs) clean and disinfect facilities and are responsible for executing infection control protocols. ESWs are often in patient areas with a similar frequency as some clinical staff, but very little research exists on their infection risks (21). A recent study found prevalence of

COVID-19 among cleaning staff in a clinic to be higher than that of clinical staff, which points to a potential undetected disease burden in ESWs (21). The US Department of Labor reports that janitors and cleaning staff have a higher incidence of illness and injury than registered nurses (23).

Significant resources have been dedicated to assessing knowledge, providing education, and changing behaviors among ESWs to improve cleaning and disinfection practices (24-27). There is evidence that integrating ESWs into the planning of infection control procedures results in reduced nosocomial infections among patients (11,28). However, current research fails to examine the health burden placed on ESWs who work in close contact with ill individuals and contaminated environments.

The majority of healthcare-associated HuNoV outbreaks begin due to environmental contamination, and fomites represent a significant environmental route of exposure (29,30,11,31-33). Despite the concerns with fomite transmission of HuNoVs, there is evidence that healthcare workers lack knowledge about fomites as a pathogen vector and may not follow appropriate hand hygiene practices after fomite contact (34,35). When focusing on ESWs, the role of fomite transmission of HuNoVs becomes even more important as cleaning activities result in a high number of fomite contacts (36). ESWs are also

likely to underestimate the risks posed to them from fomites and may be less likely to follow hand hygiene practices after contacting fomites (24).

To quantify the risks faced by ESWs during HuNoV outbreaks, a quantitative microbial risk assessment (QMRA) is necessary. QMRA is a technique to model infection and illness risk in a population exposed to microorganisms, based on a four-step process: hazard identification, exposure assessment, dose-response, and risk characterization (37). It allows for the quantification of infection risks under multiple scenarios, which can be useful in estimating health burdens, designing intervention strategies, and identifying research needs. Each step requires detailed analysis of a scenario of interest and requires quantitative information to develop a final model. In QMRA, it is possible to integrate both variability, which describes inherent differences in the population of interest, and uncertainty, which reflects imperfect knowledge (38). In general, uncertainty can be reduced with additional data and improved models, while variability will not change with more measurements, though it may be better characterized (38). Analysis of both variability and uncertainty in a QMRA can be achieved through the use of two-dimensional Monte-Carlo (2D MC) (39).

The goal of this work was to use a 2D MC method to quantify HuNoV infection risk resulting from a single fomite contact within patient areas occupied by

HuNoV positive patients who are actively shedding HuNoV. This work is one of the first to develop a QMRA for HuNoV transmission from fomites in healthcare facilities. Our model considered illness in ESWs as an endpoint, in addition to secondary illness as a result of fomite transfer from ESWs.

2 Methods

2.1 Model Scenarios

Eight scenarios were developed to compare HuNoV infection risk from different HuNoV sources, locations, and populations experiencing the health outcome (Table 1). For each scenario we aimed to develop a QMRA and 2D MC model that was protective of public health by modeling high exposure values that were plausible based on current literature. We quantified the risk of HuNoV infection from a single fomite contact that occurred after a single HuNoV source event – either vomit or diarrhea from an infected patient (Figure 1). We modeled multiple scenarios to examine risk differences between the type of source event and the location of the source event, either in the patient's bathroom or in their main room. Additionally, we were interested in two populations for the final health outcome of HuNoV infections – (1) ESWs who directly touch contaminated fomites and (2) other healthcare workers who touch a shared surface that was first touched by an ESW after their exposure to a contaminated patient's space

(“secondary fomite infections”). In all scenarios, the initial fomite contact is made by an ESW.

In Table 1, scenario number indicates the source event and location, while scenario letter refers to the population of interest for HuNoV infection. Scenario 1 modeled a patient vomiting in the bathroom, scenario 2 modeled a patient having diarrhea in the bathroom, scenario 3 modeled a patient vomiting in their room, and scenario 4 modeled a patient having diarrhea in their room. For each numerical scenario, there is an “a” and “b” scenario reflecting target health outcome – either ESWs develop a HuNoV infection (outcome a) or the contamination of shared ward fomites leads to infection in persons who contact those fomites (outcome b, “secondary fomite infections”). For in-bathroom scenarios, both source events were assumed to occur in the toilet, while for in-room events we assumed the use of a bed pan for diarrhea and a basin for vomiting. The touch fomite of interest in bathroom scenarios was the bathroom door handle and for in-patient room scenarios the fomite of interest was the patient bed rail. For secondary fomite infections, the contact surface was modeled as a door handle in common space on the ward. Model assumptions are listed in Table 2.

2.2 Modeling Approach

A 2D MC approach was used to model variability and uncertainty of inputs. The R package “mc2d” was used to build and evaluate all models (39). Distributions for inputs were designated as variability (inherent population differences), uncertainty (result of imperfect knowledge), or a combination of both. In some instances, fixed values were used for inputs. For inputs that were defined only by variability, distributions were randomly sampled to provide final estimates. For inputs defined by both uncertainty and variability, we first sampled uncertain parameters. Then, any uncertain parameters were carried into a variability distribution and randomly sampled. The final model resulted in risk estimates that reflect variability across the population, conditional upon uncertainty parameters. Model iterations were set at 1000 for the variability dimension and 100 for uncertainty. Stability of measurements was tested by running 10 different seed values. For each scenario, different seed values resulted in overlapping standard deviation intervals for each median risk estimate (data not shown).

2.3 Model Parameters

All parameters, distributions, and sources used in our models are shown in Table 3. The corresponding variable notation listed in Table 3 is provided parenthetically in text and corresponds to variable names in the R code provided as supplementary material; variables are presented in the order they are used in

corresponding R code. In general, uncertainty distributions were truncated at minimum and maximum values of corresponding variability distributions, and variability distributions were truncated at biologically impossible values (ex. stool production below zero, transfer proportions greater than 1).

2.4 Norovirus Shedding

The mean concentration of HuNoV in vomit (*m.vv*) was modeled as an uncertainty distribution using data from human challenge studies. We focused on one study that infected patients with GII.2 snow mountain virus and measured a mean shedding of 1.6×10^5 HuNoV genome equivalents (GE)/ mL vomitus with a standard deviation of 4.5×10^4 GE/ mL (40). This study was selected because it used a genotype II HuNoV, which is widely circulated in healthcare facilities (5,31). The mean virus concentration in vomit was modeled using a normal distribution. Based on a previously conducted QMRA, we then chose to model variability in norovirus concentration in vomit (*c.vv*) using a BetaPert distribution with a mode selected from the uncertainty distribution, a minimum of 2,200 GE/ mL, and a maximum of 1.2×10^7 GE/ mL (41,42).

The next parameter modeled was the volume of vomit produced in a single event. We define a vomit event as the production of vomitus that occurs within a 15-minute period (40). We assume that the vomit event is early in the HuNoV

disease course and not later when dry-heave or retching events may be more likely. Previous literature indicates that vomitus greater than 800 mL is considered abnormally high for norovirus patients and less than 50 mL is considered a “dry-heave” event (43). In HuNoV challenge studies, mean production of vomit during an entire course of infection with HuNoV GII.2 snow mountain resulted in an average of 845 mL of vomitus with a standard deviation of 227 mL and patients experienced an average of 2 vomit events through the course of their infection (40). To model uncertainty of the mean volume of vomit (*m.vlv*) from a single event, total volume of vomit was modeled with a normal distribution with mean 845 mL and standard deviation of 227 mL, then this value was divide by two to obtain estimated mean volume of vomit for one event. The resulting estimated mean was used as the mode for a BetaPert distribution with a minimum of 50 mL and maximum of 800 mL to model variability (*vl.v).*

Mean mass of feces in one diarrhea event (*m.mf*) was estimated using mean diarrhea amounts from Read et al. (44) to obtain an average daily mass of 437 g with standard deviation of 76 g. These values were divided by the estimated average number of daily bowel movements for a norovirus patient (4.45) (45). This resulted in a normal distribution with mean of 98.2 g and standard deviation of 17.08 g. The final mass of feces (*m.f*) was modeled using a BetaPert distribution with a range of 14.6 to 449.4 g which represent minimum and maximum daily mass, divided by an estimated 4.45 daily bowel movements (45).

Concentration of norovirus in feces (*c.vf*) was modeled as a variability parameter with a BetaPert distribution using values from a previous QMRA (42).

2.5 Aerosolization and Fomite Deposition

For the amount of virus released into room air, two parameters were modeled: the proportion of virus aerosolized during vomiting and the proportion aerosolized during flushing a toilet. These two values were assumed to be additive and degradation of virus in vomitus was not considered. Additionally, we assumed that diarrhea events only produced aerosol from flushing and not during defecation as individuals will block air movement while seated.

Particles released from vomit and flush events were considered to reach their maximum number in one m^3 space around the toilet as research has indicated an initial dispersion of particles post-flush of approximately 1 m above the ground (46). Particles were subsequently assumed to evenly disperse through the room within 1 minute (47). The total amount of virus per m^3 was calculated by dividing total particles aerosolized by room volume (46).

To calculate the norovirus aerosolization from a vomit event (*p.av*) we used data reported by Tung-Thompson et al. (43). We chose values from a high pressure

simulated vomiting event with low viscosity vomitus, as these values reflect a worst case scenario. Variability in the proportion of particles aerosolized from one vomit event was modeled as a normal distribution based on concentration of virus in vomit – for low titer vomitus ($<10^{10}$ GE) we used a mean of 2.8×10^{-5} and a standard deviation of 1×10^{-5}, while for high titer vomitus ($\geq 10^{10}$ GE) we used a mean of 1.3×10^{-4} and a standard deviation of 1×10^{-4}. We tested cutoff values from 10^{7} to 10^{10} for low versus high titer vomitus and no difference was observed in final risk estimates (data not shown).

For variability in aerosolization of norovirus from toilet flushing (*p.af*) we used data from Barker and Jones (48) where a toilet bowl was inoculated with a known amount of the HuNoV surrogate MS2, flushed, and air concentration was measured. For one flush, 2.42×10^{-7} of in-toilet virus was aerosolized and this was modeled as the mean of a normal distribution with a standard deviation of 6.91×10^{-8}.

Bathroom volume was calculated from a study that measured areas of bathrooms in a modern tertiary care hospital (49). The height of the room was assumed to be 2.39 m, which meets Facility Guidelines Institute (FGI) guidelines for hospital construction (50). Area values were multiplied by 2.39 and values were averaged to obtain an estimated bathroom volume of 12.4 m^3. For patient

room volumes, suggested room areas from the literature were multiplied by an assumed room height of 2.39 m and averaged, resulting in an estimated patient room volume of 26.5 m^3 (51). It was assumed that patient room doors and bathroom doors were closed during source events and that air mixing between the rooms did not occur. We then calculated the concentration of HuNoV per m^3 in the room air for each scenario using amount of virus aerosolized from vomit event and/ or toilet flush.

Equations for each scenario were:

Vomit in Bathroom, Immediately Post Flush (s1a, s1b)

*(c.vv * vl.v * (p.av + p.af)) / vlr* *[c.va1]*

Diarrhea in Bathroom, Immediately Post Flush (s2a, s2b)

*(c.vf * m.f * p.af) / vlr* *[c.va2]*

Vomit in Patient Room, Immediately After Event (s3a, s3b)

*(c.vv * vl.v * p.av) / vlr* *[c.va3]*

For scenarios 4a and 4b (diarrhea in patient room) we assumed no aerosolization due to the absence of both vomiting and toilet flushing.

To model the settling rate of aerosolized virus on to fomites, we used air sampling and settle plate data from Barker and Jones (48). After flushing a toilet

inoculated with MS2, the authors measured a 93% reduction in virus particles in air after 30 minutes post-flush. We estimated that 93% of virus in a m^3 area will fall out on a m^2 in 30 minutes, which results in a fomite deposition rate of 0.031 from m^3 to m^2 per minute, for up to 30 minutes. After 30 minutes, we assumed that 100% of aerosolized particles deposited on to fomites. We modeled time since event (*delt.t*) as a uniform distribution from 1 to 1440 minutes, assuming an ESW would enter a room once per day for cleaning (52).

Surface area of hand contact (*ah*) was estimated by painting a standard metal lever-type door handle, having author KO grab the handle to simulate opening a door, and measuring the surface area of paint transferred to a sheet of paper. This value was 0.001268 m^2 and was compared to data on average human hand size from the EPA Exposure Factors Handbook to verify that our value was plausible (53). We assumed that hand-fomite contact area was constant for both fomites used in our scenarios - door handles and bed rails.

To obtain estimates of finger pad area (*af*) we used a study by Dandekar et al. that found the average width of an adult index finger to range from 16 - 20 mm and we assumed a square finger pad area for a resulting uniform distribution that ranged from 0.000256m^2 to 0.0004m^2 (54). To calculate virus particles on finger pads the concentration of particles on the hand was multiplied by the ratio

between hand touch area and finger pad (*r.fh*).

2.6 Transfer Efficiencies

To calculate the proportion of feces transferred to hands during wiping (*t.fh*), we used previous data on mass of feces transfer to hands and our estimate of 98.2 g mean stool per event to model this value as a BetaPert distribution with a mode of 10^{-6}, minimum of 10^{-10}, and maximum of 10^{-3} (42). Transfer of HuNoV particles from hands to fomites and from fomites to hands were estimated using data from Julian et al. (55). We chose reported transfer values for the surrogate virus MS2 due to its similar size and surface structure compared to HuNoV. We also used values reported for recently washed hands under the assumption that in a hospital setting recent hand washing is likely to have occurred. This resulted in a normal distribution with a mean of 0.15 and a standard deviation of 0.16 for transfer of virus from hands to fomites (*t.hs*) and a normal distribution with a mean of 0.26 and a standard deviation of 0.19 for transfer of virus from fomites to hands (*t.sh*). Transfer of virus particles from finger pad to mouth (*t.hm*) was modeled with a normal distribution with a mean of 0.34 and a standard deviation of 0.25 as reported previously (56-59).

2.7 Fomite and Hand Concentrations

Equations to calculate total amount of HuNoV deposited on touch area were:

Vomit in Bathroom, Immediately Post Flush (s1a, s1b)

*s.as * c.va * ah* *[c.vs1]*

Diarrhea in Bathroom, Immediately Post Flush (s2a, s2b)

*(s.as * c.va * ah) + (m.f * t.fh * c.vf * t.hs)* *[c.vs2]*

Vomit in Patient Room, Immediately After Event (s3a, s3b)

*s.as * c.va * ah* *[c.vs3]*

Diarrhea in Patient Room, Immediately After Event (s4a, s4b)

*m.f * t.fh * c.vf * t.hs* *[c.vs4]*

For vomit scenarios (1a, 1b, 3a, 3b) we assumed no hand transfer of virus to fomites occurred.

We then used the concentration of virus particles on a fomite to calculate the number of virus particles on an ESW's hands after contact with the fomite:

*c.vs * t.sh* *[c.vh]*

We also calculated the number of particles an ESW would transfer to secondary contact fomites (for all "b" scenarios):

*c.vh * t.hs* *[c.v2s]*

2.8 Dose

Norovirus dose was considered the amount of virus particles transferred from a finger pad to an ESW's mouth ("a" scenarios) or secondary contact's mouth ("b" scenarios). We chose to model ingestion as result of finger pad contact because previous work has found that 90% of touches to mucous membranes are by fingers (60). We assumed that transfer from finger pad to mouth resulted in ingestion of 100% of transferred particles. This resulted in the following dose equations:

Norovirus Dose for ESW (1a, 2a, 3a, 4a)

*c.vh * r.hf * t.hm* *[D]*

Norovirus Dose for Secondary Fomite Contact (1b, 2b, 3b, 4b)

*c.vs2 * t.sh * r.hf * t.hm* *[D2]*

2.9 Dose Response and Endpoint

The endpoint for this risk model was HuNoV illness in either an ESW or a secondary fomite exposed contact. We chose a previously reported dose-

response model to estimate probability of illness, with parameters n and r that take the values 2.55 x 10^{-3} and 0.086, respectively (61,18):

*p(ill/dose) = 1 - (1 + n*dose))^-r* *[risk.ill]*

2.10 Sensitivity Analysis

A sensitivity analysis was conducted by calculating the Spearman rank correlation coefficient (SRC) for each baseline parameter. SRC values can range from -1 to 1 and an |SRC| value closer to 1 indicates higher correlation and higher importance of the factor on the final risk value. Baseline parameters were defined as values that were supplied to the model as distributions and not calculated within the model. Values that were supplied as constants were excluded from sensitivity analyses. Baseline parameters evaluated in the final sensitivity analysis were concentration of norovirus in vomit (*c.vv*), volume of vomit (*vl.v*), concentration of norovirus in feces (*c.vf*), mass of feces (*m.f*), proportion of virus aerosolized from vomit and toilet flush (*p.av*, *p.af*), settling rate of norovirus from air (*s.as*), time between source event and fomite contact (*delt.t*), area of finger pad (*af*), and transfer or norovirus from feces to hands (*t.fh*), from hands to fomites (*t.hs*), from fomites to hands (*t.sh*), and from hands to mouth (*t.hm*).

3 Results

Median risk estimates and 95% credible intervals for a single fomite contact are shown in Table 4. Risk values for diarrhea scenarios (s2, s4) were the same when compared across the two locations (bathroom and patient room). Median HuNoV infection risk for diarrhea source events was calculated to be 1:3 for ESWs whether they were exposed in the bathroom or in the patient room and 1:4 for secondary contacts. Risk values were lower for vomit scenarios and ranged from a 1:23,928 median risk of ESW illness following a vomit event in a patient's bathroom to 1:252,185 median risk for a secondary contact after a vomit event in a patient room. Median infection risks from a vomit event were higher in the scenarios where the source event occurred in the bathroom (s1), as compared to the patient room. Secondary transmission of HuNoV by ESWs resulted in an 80% decrease in median infection risk for vomit scenarios (s1, s3) and a 32% decrease for diarrhea scenarios (s2, s4).

SRC values from the sensitivity analysis for baseline parameters in each scenario are shown in Figure 2. Settling rate of norovirus from air to fomites had the lowest SRC in all scenarios where it was included. In vomit scenarios (s1, s3) the starting concentration of HuNoV in vomit had much less effect on the final risk model, compared with the impact of concentration of norovirus in diarrhea (s2, s4). In scenario 1, the value for aerosolization from vomit had equivalent

SRC values compared to aerosolization from toilet flush. In all scenarios with an aerosolization component (s1, s2, s3) the time between event and fomite contact was strongly correlated to the final risk model (SRC > 0.8). All modeled transfer rates had SRC values of 0.8 or higher and in diarrhea scenarios transfer rates were significantly correlated with final risk values (SRC 0.99). (add an interpretation sentence here)

4 Discussion

This work is the first to use a QMRA to model infection risk of HuNoV in ESWs from fomite contacts in healthcare settings. For a single fomite contact in a room where a patient experienced a diarrhea event, the risk of HuNoV infection for ESWs was as high as 1:3. Though high, this risk estimate is in line with previous reports of HuNoV attack rates in staff between 30% and 90% in hospital outbreaks (9,19,14). For scenarios where the ill patient vomited without a diarrheal event, the estimated risk to ESWs from fomite contact dropped significantly to 1:23,928 for an in-bathroom event and 1:51,504 for an in-room event. It is important to emphasize that these risk estimates represent infection risk from a single contact event with a single fomite. Data indicates that during cleaning, an average of 9 fomites are touched per room visit, with a maximum of 34 (36). If risks from a single fomite are additive, infection risks from a single room visit could be significant.

We found much higher risks of infection from diarrhea source events, compared to vomit events. The impact of HuNoV transmission from diarrhea versus vomit events remains unclear in the literature. One study reported that vomiting patients infect twice the number of people as those who are not vomiting, while patients with diarrhea infect 1.4 times the number of people as those without diarrhea (62). However, another study indicates that diarrhea is almost ubiquitous in index cases for HuNoV outbreaks and that diarrhea had a higher association with outbreak development, compared to vomiting (63). One reason for the low estimated risks from vomit source events is the focus on just the fomite route of transfer and the exclusion of any inhalation of viral particles. Previous literature has indicated aerosol transmission of HuNoV and vomiting appears to be the main source of HuNoV aerosols (64,65). Aerosolization of HuNoV is thought to be highest right after vomiting and presence of HuNoV in the air ablates after 3-6 hours post vomit event (64). Our work demonstrates that even after reduction of virus aerosolization, fomite exposure to HuNoV can remain a significant source of infection. Additionally, risk for fomite exposure may actually increase with time since event, which can significantly affect long-term exposure to the virus. Add thing about ESWs here

For secondary HuNoV infection from contact with a contaminated fomite, risks were 32% lower than ESW infection risks in diarrhea scenarios and 80% lower than ESW infection risks in vomit scenarios. This aligns with evidence that proximity to HuNoV patients increases the likelihood of viral spread in healthcare (66). The infection risks faced by secondary fomite contacts are affirmed by previous work that shows spread of viral surrogates between multiple rooms and fomites in a hospital (67,32).

Our sensitivity analyses indicated that the concentration of HuNoV in vomit was much less important in relevant scenarios, when compared to the concentration of HuNoV in diarrhea. This is likely due to overall less transmission of HuNoV via fomites in vomit scenarios. We also found that a longer time between the source event and ESW contact led to a larger risk. This makes sense with the current model because we did not model die off and a longer time period would allow more virus to settle. However, this finding likely only holds true in scenarios where no air inhalation can occur, which is not the case for HuNoV.

We also found that transfer rates were a significant driver of infection risk in our models, especially in diarrhea scenarios. This serves to underscore the need for more robust and specific data on transfer of HuNoV across different fomites. Lack of these data contribute to a significant gap in our understanding of

healthcare-associated outbreaks of HuNoV (68,69).

This work was subject to several limitations. Scenarios only accounted for HuNoV transfer from patients with active symptoms. Asymptomatic shedding of HuNoV is well documented, but literature indicates that symptomatic patients remain the main drivers of transmission in healthcare settings (70). We chose not to examine the role of hand hygiene or other intervention measures in this model. One reason for this was we aimed to capture a worst-case scenario of transmission. Further, there is evidence of poor hand hygiene compliance among ESWs after fomite contact (36). Evidence also exists that HuNoV persists on fomites even after cleaning and transmission of HuNoV in healthcare settings is likely to occur even in the presence of interventions (71,20,31). Our models did not include any die off of HuNoV, which is justified by literature indicating persistence of HuNoVs on fomites well past the 24-hour time span examined in this work (72,69,73). However, our model also did not assume any additive effect from previous HuNoV source events, though this is likely to occur in real-world settings. We assumed that all HuNoV produced in the source event was infectious, when it is likely that a subset of measured HuNoVs are non-infectious particles. The recent development of a cell culture model for HuNoV represents an exciting opportunity to further refine these data (4,74).

We were unable to model all parameters using HuNoV-specific values and to separate out values for different HuNoV genotypes and genogroups. In particular, transfer rates for feces to hands during wipe were challenging to find in existing literature. Previous studies have addressed this by assuming a mass transfer of 0.1 g (75), while another study assumed a BetaPert distribution with a mode of 0.001 g feces, a minimum of 1×10^{-8} g, and a maximum of 1×10^{-1} g, though rationale for these values was not provided (42). When reported mass transfer from these studies was combined with our assumed mean stool weight of 98.2 g, the mode percent transfer of feces to hands was 0.1% (75) and 0.001% (42). A third study measured transfer of the surrogate feline calicivirus (FCV) to hands from artificial feces and found that approximately 3% of virus was transferred in a high-contact scenario (76). These values result in feces to hand transfer rates that span four orders of magnitude. A similar variability was observed in literature on aerosolization of particles from a toilet flush. The generation rates calculated in this work were similar to that found by Johnson et al. (47) who estimated approximately 0.072 droplets forming for every 100 million particles; resulting in an aerosolization proportion of 1.3×10^{-10}. However, another study estimated that between 33.3% and 60% of the total particles in a toilet rise above the toilet seat during a flush event (46). This results in possible aerosolization proportions from a toilet flush that differ by over 10 orders of magnitude.

This study adds to the growing body of literature that points to fomite transmission as a significant pathway in the spread of HuNoVs (77,72,78,73,79). We show that it is feasible for a single fomite contact to lead to a sizable risk of HuNoV infection in ESWs and that ESWs are able to transmit a significant amount of virus to secondary fomites, initiating further infections. Our work highlights the importance of studying ESWs as a unique population in healthcare settings, independent of clinical staff. ESWs are the first line of defense against healthcare associated infections, yet our understanding of the health risks they face remains minimal. This QMRA shows that ESWs likely face important occupational health risks from fomite-mediated infections and interventions are needed to both protect them and prevent further spread. Fomite cleaning and disinfection procedures should be designed in collaboration with ESWs and future work must focus on quantifying the risks posed to these essential workers.

Acknowledgements

The authors would like to thank Kerry Hamilton and Emily Cooksey at Arizona State University for technical assistance.

Tables

Table 4.1 Model scenario by human norovirus source event, contact setting, and outcome

	Vomit		Diarrhea	
	Environmental service worker becomes ill	Ward Transmission	Environmental service worker becomes ill	Ward Transmission
In Bathroom	1a	1b	2a	2b
In Patient Room	3a	3b	4a	4b

Table 4.2 Major assumptions of the models

Assumptions Related to HuNoV Symptoms and Shedding
All particles released from ill patient are initially infectious
Source of virus is either bowel movements or vomit events – the patient does not passively shed
Assumptions Related to Aerosolization of Viral Particles
Bathroom door is closed during vomit/ diarrhea event
Air mixing between rooms does not occur
Air distribution of viral particles post flush and/ or post vomit event is uniform and occurs within 1 minute
No aerosolization occurs directly from a diarrheal event
Assumptions Related to Fomite Deposition of Viral Particles
Virus in air does not settle on hands, it only deposits on fomites
Deposition of virus from air to fomites occurs uniformly across the room
Assumptions Related to Transfer of Viral Particles from Contact
ESW cleans room once a day
Virus does not inactivate in air or on fomites
Transfer of virus from the hands of ill patient does not occur during vomit events and only results from wiping after a diarrhea event
Transfer of viruses between fomites and hands is one directional - moving from fomite with high concentration to fomite with low concentration
Distribution of virus is constant across touch area
Viral transfer rates are the same for each fomite

Assumptions Related to Calculated Fomite Concentration and Dose of HuNoV

Contact between hand and mouth is done via single finger pad

Only one hand to face contact event occurs before handwashing

Dose to lip is considered ingestion

Table 4.3 Model parameter distributions and sources

Description	Units	Distribution (values)[a]	Truncation	Type[b]	Source
Virus shedding					
m.vv = mean concentration of HuNoV in vomitus	genome equivalents /mL	Normal (1.6 x 10^5, 4.5 x 10^4)	Min = 2200 Max = 1.2 x 10^7	U	(40)
c.vv = starting concentration of HuNoV in vomitus	genome equivalents /mL	BetaPert (2.2 x 10^3, m.vv, 1.2 x 10^7)	Min = 0	VU	(41,42)
m.vlv = mean volume of vomit in one event	mL	Normal (845, 226.7)	Min = 500 Max = 1000	U	(80,43)
vl.v = volume of vomit in one event	mL	BetaPert (50, (m.vlv/2), 800)	Min = 0	VU	(40,80,43)
m.mf = mean mass of feces from one event	grams	Normal (98.2, 17.08)	Min = 14.6 Max = 449.4	U	(45,44)
m.f = mass of feces from one event	grams	BetaPert (14.6, m.mf, 449.4)	Min = 0	VU	(45,44)
c.vf = starting concentration of HuNoV in feces	genome equivalents/ grams	BetaPert (10^4,10^8,10^{10})	Min = 10^4 Max = 1.6 x 10^{12}	V	(17,81,42)
Aerosolization					

p.av= proportion of virus particles released into air during vomit event	proportion	*Low titer (c.vv < 10^{10}):* Normal (2.8 x 10^{-5}, 1 x 10^{-5}) *High titer (c.vv ≥ 10^{10}):* Normal (1.3 x 10^{-4}, 1 x 10^{-4})	Min = 0 Max = 1	V	(65,43)
p.af = proportion of virus particles released into air during flush	proportion	Normal (2.42 x 10^{-7}, 6.91 x 10^{-8})	Min = 0 Max = 1	V	(48)
vlr = volume of room	m^3	*Bathroom (s1a, s1b, s2a, s2b):* 12.4 *Patient Room (s3a, s3b, s4a, s4b):* 26.5	-	V	(51,49,50)
c.va = concentration of virus in room air	virus particles/ m^3	*Vomit in Bathroom, Immediately Post Flush (s1a, s1b)* (c.vv * vl.v * (p.av + p.af)) / vlr *Diarrhea in Bathroom, Immediately Post Flush (s2a, s2b)* (c.vf * m.f * p.af) / vlr *Vomit in Patient Room, Immediately After Event (s3a, s3b)* (c.vv * vl.v * p.av) / vlr	-	V	Calculated

		Diarrhea in Patient Room, Immediately After Event (s4a, s4b)			
		0			
Fomite deposition					
delt.t = time between event and HCW fomite contact	minutes	Uniform (1,1440)	-	V	Assumed
s.as = virus settling rate from air to fomite during total time since event	proportion/ m^2	≤ 30 minutes since event: 0.031 * delt.t > 30 minutes since event: 1	-	V	(48)
ah = area of hand that contacts fomite	m^2	0.001268	-	0	This study
af = area of finger pad	m^2	Uniform(0.000256, 0.0004)	-	V	(54,58)
r.fh = ratio between area of hand touch area and finger pad	m^2	af / ah	-	-	Calculated
Transfer Efficiencies					
t.fh = proportion of feces transferred to hands during wiping	proportion	BetaPert (10^{-10}, 10^{-6}, 10^{-3})	Min = 0 Max = 1	V	(42,75,76)
t.hs = transfer of virus particles from hand to fomite	proportion	Normal (0.15, 0.16)	Min = 0 Max = 1	V	(55)

t.sh = transfer of virus particles from fomite to hand	proportion	Normal (0.26, 0.19)	Min = 0 Max = 1	V	(55)
t.hm = transfer between hand and mouth	proportion	Normal (0.34, 0.25)	Min = 0 Max = 1	V	(56-59)
Fomite and Hand Concentrations					
c.vs = concentration of virus particles on contact area at time t	virus particles	*Vomit in Bathroom, Immediately Post Flush (s1a, s1b)* s.as * c.va * ah *Diarrhea in Bathroom, Immediately Post Flush (s2a, s2b)* (s.as * c.va * ah) + (m.f * t.fh * c.vf * t.hs) *Vomit in Patient Room, Immediately After Event (s3a, s3b)* s.as * c.va * ah *Diarrhea in Patient Room, Immediately After Event (s4a, s4b)* m.f * t.fh * c.vf * t.hs	-	-	Calculated
c.vh = concentration of virus on ESW hands	virus particles	c.vs * t.sh	-	-	Calculated

c.v2s = concentration of norovirus on secondary contact fomite	virus particles	c.vh * t.hs	-	-	Calculated
Dose					
D = dose of virus ingested from primary fomite contact	particles	c.vh * r.hf * t.hm	-	-	Calculated
D2 = dose of virus ingested from secondary fomite contact	virus particles	c.vs2 * t.sh * r.hf * t.hm	-	-	Calculated
Dose response					
n = dose response constant	-	0.00255	-	-	(61,18)
r = dose response constant	-	0.086	-	-	(61,18)
risk.ill = risk of illness as a function of dose	risk	1 - (1 + n*dose))^-r	-	-	(61,18)

[a] Distribution types and values: Normal (mean, sd); BetaPert (minimum, mode, maximum); Uniform (minimum, maximum); Empirical (observed values)

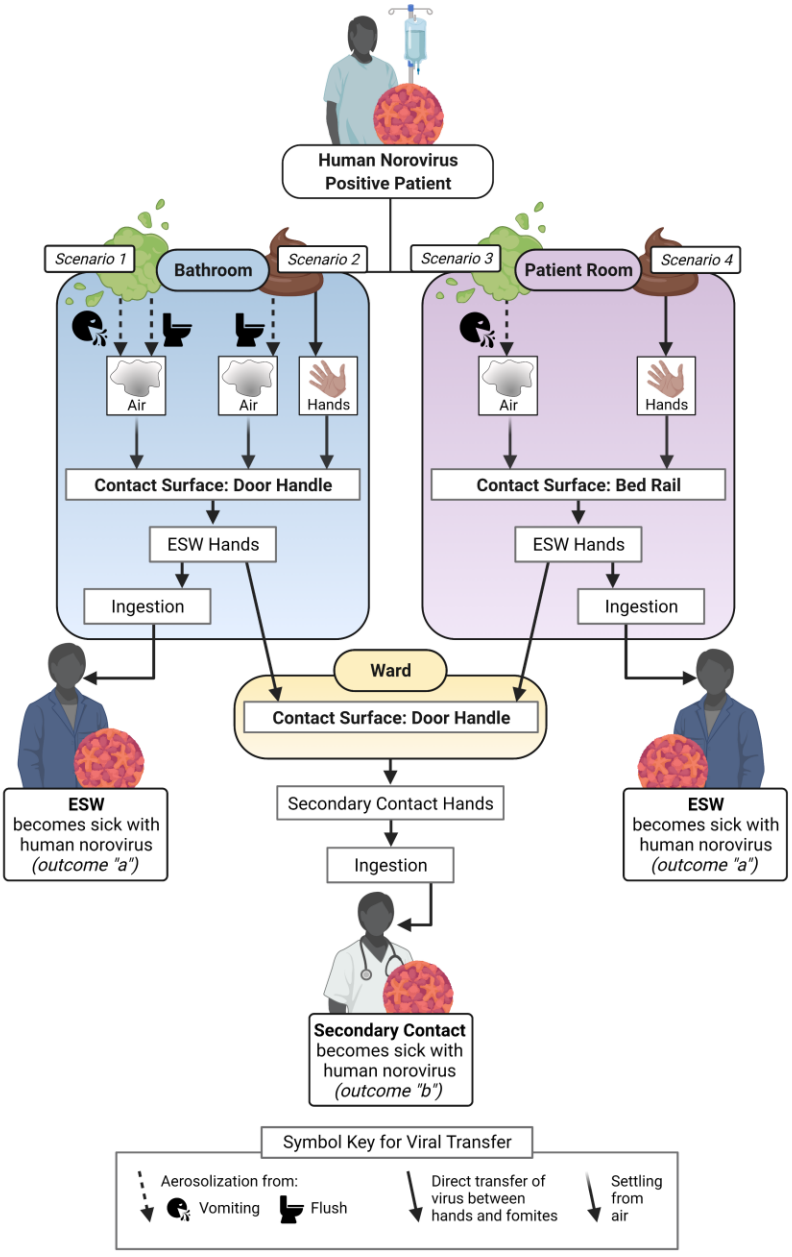
[b]U indicates uncertainty distribution, V indicates variability distribution, U + V is distributions with both an uncertainty and variability component

Table 4.4 Human norovirus infection risk for each scenario

Scenario	Median Risk of Illness (ratio of illness: touch events)	95% Confidence Interval for Risk of Illness	Minimum Risk Estimate	Maximum Risk Estimate
1a	4.18×10^{-5} (1:23,928)	(3.32×10^{-5}, 5.02×10^{-5})	1.40×10^{-14}	1.15×10^{-3}
1b	8.54×10^{-6} (1:117,104)	(6.50×10^{-6}, 1.07×10^{-5})	$< 1 \times 10^{-16}$	6.02×10^{-4}
2a	3.46×10^{-1} (1:3)	(3.38×10^{-1}, 3.53×10^{-1})	1.80×10^{-14}	6.84×10^{-1}
2b	2.35×10^{-1} (1:4)	(2.27×10^{-1}, 2.41×10^{-1})	$< 1 \times 10^{-16}$	6.69×10^{-1}
3a	1.94×10^{-5} (1:51,504)	(1.54×10^{-5}, 2.33×10^{-5})	6.27×10^{-15}	5.36×10^{-4}
3b	3.97×10^{-6} (1:252,185)	(3.02×10^{-6}, 4.97×10^{-6})	$< 1 \times 10^{-16}$	2.80×10^{-4}
4a	3.46×10^{-1} (1:3)	(3.38×10^{-1}, 3.53×10^{-1})	1.79×10^{-14}	6.84×10^{-1}
4b	2.35×10^{-1} (1:4)	(2.27×10^{-1}, 2.41×10^{-1})	$< 1 \times 10^{-16}$	6.69×10^{-1}

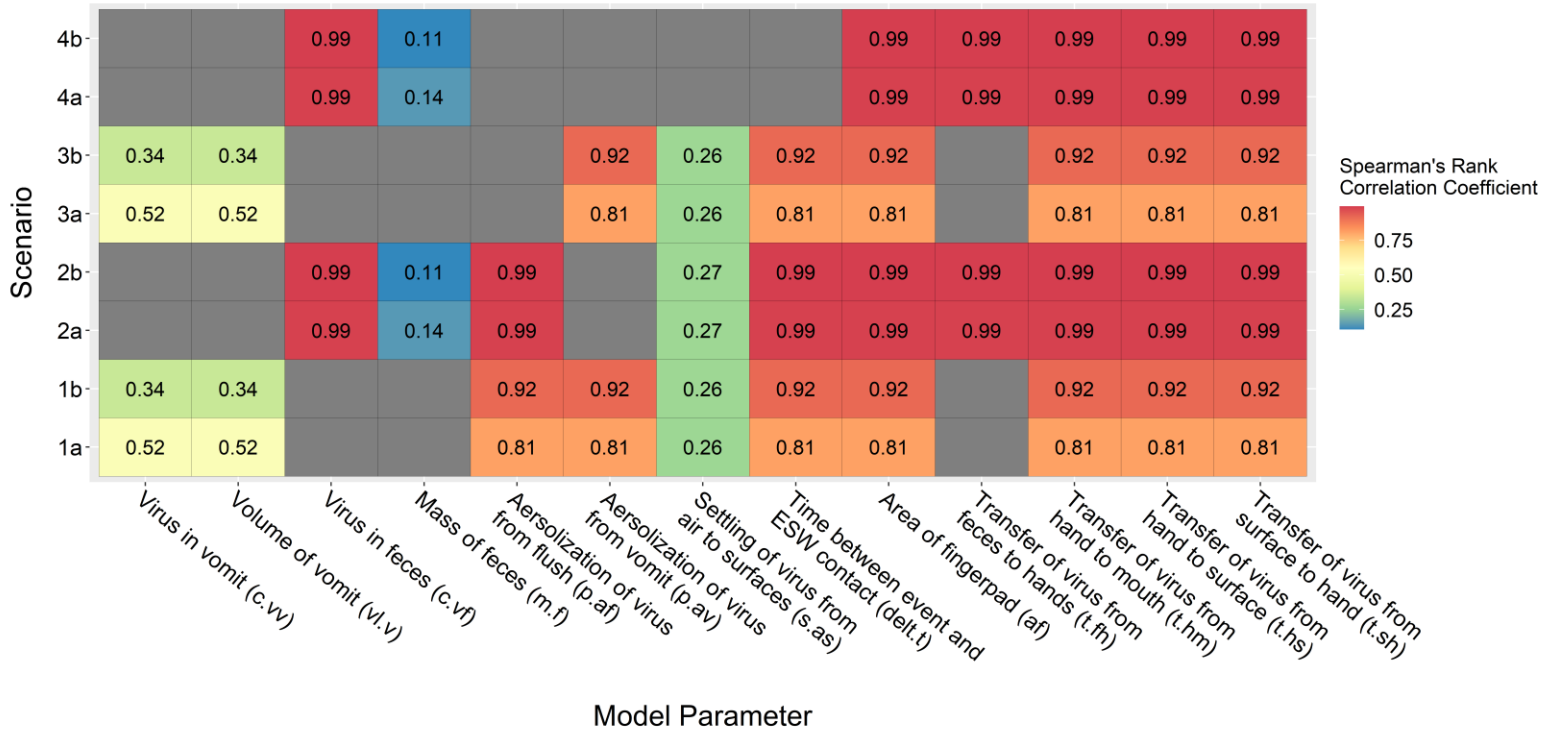
Figures

Figure 4.1 Conceptual exposure model for human norovirus transmission in each scenario



Created with BioRender.com

Figure 4.2 Spearman's rank correlation coefficient for baseline parameters in each modeled scenario



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Chapter 4 Supplementary Material: R Code for Quantitative Microbial Risk Assessment

```r
#Packages needed:
library(triangle)
library(readr)
library(expm)
library(ggplot2)
library(scModels)
library(mc2d)
library(tidyverse)
library(readxl)
library(haven)
library(ggsignif)
library(ggplot2)
library(ggpubr)
library(ggthemes)
library(reshape2)
library(gridExtra)
library(EnvStats)
library(dplyr)
library(scales)
library(Rmisc)
library(gridExtra)
```

```
library(FSA)
library(jsonlite)
library(curl)
library(sysfonts)
library(doBy)
library(data.table)
library(sfsmisc)
library(scales)
library(grid)

ndvar(1000)
ndunc(100)
seed=323

# VIRUS SHEDDING-----------------
#m.vv Mean conc virus in vomit (log particles per mL):
m.vv <- mcstoc(rnorm, type="U", mean=160000, sd=45000, seed=seed,
rtrunc=TRUE, linf=2200, lsup=12000000)

# c.vv  Conc virus in vomit (particles/mL):
c.vv <- mcstoc(rpert, type="VU", min=2200, mode=m.vv, max=12000000,
seed=seed, rtrunc=TRUE, linf=0)

# m.vlv Mean volume of vomit (log mL):
```

```
m.vlv <- mcstoc(rnorm, type="U", mean=845, sd=226.7, seed=seed,
                    rtrunc=TRUE, linf=500, lsup=1000)

# vl.v Volume of vomit in one event (mL):
vl.v <- mcstoc(rpert, type="VU", min=50, mode=(m.vlv/2), max=800,
seed=seed, rtrunc=TRUE, linf=0)

#mm.f.log Mean mass of feces in one event (g)
m.mf <- mcstoc(rnorm, type="U", mean=98.2, sd=17.08, seed=seed,
rtrunc=TRUE, linf=14.6, lsup=449.4)

#m.f Mass of feces from one event (g)
m.f <- mcstoc(rpert, type="VU", min=14.6, mode=m.mf, max=449.4,
seed=seed, rtrunc=TRUE, linf=0)

#c.vf Concentration of virus in feces (partilces/ g)
c.vf <- mcstoc(rpert, type="V", min=10000, mode=100000000,
max=10000000000, seed=seed, rtrunc=TRUE, linf=10000,
lsup=1600000000000)

#AEROSOLIZATION----------------------
#p.av Proportion of virus particles released into air during vomit
event (fraction):
p.av <- ifelse(c.vv<10000000000, mcstoc(rnorm, type="V",
```

```
mean=(0.000028), sd=0.00001, seed=seed, rtrunc=TRUE, linf=0, lsup=1),
                  mcstoc(rnorm, type="V", mean=0.00013, sd=0.0001,
seed=seed, rtrunc=TRUE, linf=0, lsup=1))

# p.af Proportion of virus particles rleased into air during flush
(fraction):
p.af <- mcstoc(rnorm, type="V", mean=0.000000242,  sd=0.0000000691,
seed=seed, rtrunc=TRUE, linf=0, lsup=1)

#vl.r Total volume of room
# vlr Total volume of bathroom (m^3) or patient room:

#bathroom volume:
vlr.12 <- mcdata(12.4, type="0")

#pt room (3a, 3b, 4a, 4b)
vlr.34 <- mcdata(26.5, type="0")

# c.va Concentration of virus in bathroom air (particles/ m^3)
#Vomit in Bathroom, Immediately Post Flush (s1a, s1b)
c.va1 <- (c.vv * vl.v * (p.av + p.af)) / vlr.12

#Diarrhea in Bathroom, Immediately Post Flush (s2a, s2b)
c.va2 <- (c.vf * m.f * p.af)/vlr.12
```

```
#Vomit in Patient Room, Immediately After Event (s3a, s3b)
c.va3 <- (c.vv * vl.v * p.av)/ vlr.34

#SURFACE DEPOSITION ---------------------
# delt.t Time between vomit event and HCW door knob contact (minutes):
delt.t <- mcstoc(runif, type = "V", min = 1, max = 1440, seed=seed,
rtrunc=TRUE, linf=0)

# s.as Virus settling rate from air to surfaces (fraction of particles
in m^3/ m^2/ minute):
s.as <-  ifelse(delt.t<31, mcdata(0.031*delt.t, type="V"), mcdata(1,
type="0"))

# ah Area touched by hand (m^2):
ah <-  mcdata(0.001268, type="0")

#af Area of finger pad:
af <- mcstoc(runif, type="V", min=0.000256, max=0.0004, seed=seed)

#r.hf ratio of fingerpad to hand
r.fh <- af/ah

#TRANSFER EFFICIENCIES ---------------
```

```r
#t,fh Transfer of feces to hand after wipe
t.fh <- mcstoc(rpert, type="V", min=0.0000000001, mode=0.000001,
max=0.001, seed=seed, rtrunc=TRUE, linf=0, lsup=1)

#t.hs Transfer efficiency from hand to surface
t.hs <- mcstoc(rnorm, type = "V", mean=0.15, sd=0.16, seed=seed,
rtrunc=TRUE, linf=0, lsup=1)

#t.sh Transfer efficient from surface to hand
t.sh <- mcstoc(rnorm, type="V", mean=0.26, sd=0.19, seed=seed,
rtrunc=TRUE, linf=0, lsup=1)

#t.hm  Transfer efficiency between hand and mouth (fraction):
t.hm <- mcstoc(rnorm, type="V", mean=0.34, sd=0.25, seed=seed,
rtrunc=TRUE, linf=0, lsup=1)



#SURFACE and HAND CONCENTRATIONS----------------
# c.vs Particles on door handle at time t (particles)
#1a, 1b: Vomit in Bathroom, Immediately Post Flush
c.vs1 <- (s.as * c.va1 * ah)

#2a, 2b: Diarrhea in Bathroom, Immediately Post Flush
c.vs2 <- (s.as * c.va2 * ah) + (m.f * t.fh  * c.vf * t.hs)
```

```
#3a, 3b: Vomit in Patient Room, Immediately After Event
c.vs3 <- s.as * c.va3 * ah

#4a, 4b: Diarrhea in Patient Room, Immediately After Event
c.vs4 <- m.f * t.fh  * c.vf * t.hs

# ALL c.vh Concentration of virus on hands (particles/ hand):
c.vh1 <- c.vs1 * t.sh
c.vh2 <- c.vs2 * t.sh
c.vh3 <- c.vs3 * t.sh
c.vh4 <- c.vs4 * t.sh

#c.v2s Concentration of virus on secondary contact surface (ie. ward
door)
c.v2s1 <- c.vh1 * t.hs
c.v2s2 <- c.vh2 * t.hs
c.v2s3 <- c.vh3 * t.hs
c.v2s4 <- c.vh4 * t.hs

#DOSE--------------------------
# D Dose of virus ingested by ESW:
D1 <- c.vh1 * r.fh * t.hm
D2 <- c.vh2 * r.fh * t.hm
```

```
D3 <- c.vh3 * r.fh * t.hm

D4 <- c.vh4 * r.fh * t.hm


# Dsec Dose of virus ingested by secondary contact on ward:

Dsec1 <- c.v2s1 * t.sh * r.fh * t.hm

Dsec2 <- c.v2s2 * t.sh * r.fh * t.hm

Dsec3 <- c.v2s3 * t.sh * r.fh * t.hm

Dsec4 <- c.v2s4 * t.sh * r.fh * t.hm


# Dose response constants - using Miranda 2018 paper

n <- mcdata(0.00255, type="0")

r <- mcdata (0.086, type="0")

risk.ill.1a <- 1 - (1+(n*D1))^(-r)

risk.ill.2a <- 1 - (1+(n*D2))^(-r)

risk.ill.3a <- 1 - (1+(n*D3))^(-r)

risk.ill.4a <- 1 - (1+(n*D4))^(-r)


risk.ill.1b <- 1 - (1+(n*Dsec1))^(-r)

risk.ill.2b <- 1 - (1+(n*Dsec2))^(-r)

risk.ill.3b <- 1 - (1+(n*Dsec3))^(-r)

risk.ill.4b <- 1 - (1+(n*Dsec4))^(-r)


#mc ----------------------

s1a <-  mc(m.vv, c.vv, m.vlv, vl.v, p.av, p.af, delt.t, s.as, t.hs,
```

```
t.sh, t.hm, vlr.12,
            ah, af, r.fh, c.va1, c.vs1, c.vh1, D1, n, r, risk.ill.1a)

s1b <- mc(m.vv, c.vv, m.vlv, vl.v, p.av, p.af, delt.t, s.as, t.hs,
t.sh, t.hm, vlr.12,
          ah, af, r.fh, c.va1, c.vs1, c.vh1, c.v2s1, Dsec1, n, r,
risk.ill.1b)

s2a <- mc(m.mf, m.f, c.vf, p.af, vlr.12, c.va2, delt.t, s.as, ah, af,
r.fh,
          t.fh, t.hs, t.sh, t.hm, c.vs2, c.vh2, D2, n, r, risk.ill.2a)

s2b <- mc(m.mf, m.f, c.vf, p.af, vlr.12, c.va2, delt.t, s.as, ah, af,
r.fh,
          t.fh, t.hs, t.sh, t.hm, c.vs2, c.vh2, c.v2s2, Dsec2, n, r,
risk.ill.2b)

s3a <- mc(m.vv, c.vv, m.vlv, vl.v, p.av, delt.t, s.as, t.hs, t.sh,
t.hm, vlr.34,
           ah, af, r.fh, c.va3, c.vs3, c.vh3, D3, n, r, risk.ill.3a)

s3b <- mc(m.vv, c.vv, m.vlv, vl.v, p.av, delt.t, s.as, t.hs, t.sh,
t.hm, vlr.34,
          ah, af, r.fh, c.va3, c.vs3, c.vh3, c.v2s3, Dsec3, n, r,
```

```
risk.ill.3b)

s4a <- mc(m.mf, m.f, c.vf, ah, af, r.fh, t.fh, t.hs, t.sh, t.hm,
          c.vs4, c.vh4, D4, n, r, risk.ill.4a)

s4b <- mc(m.mf, m.f, c.vf, ah, af, r.fh, t.fh, t.hs, t.sh, t.hm,
          c.vs4, c.vh4, c.v2s4, Dsec4, n, r, risk.ill.4b)




#S1A: vomit in bathroom, ESW gets sick ----------------
summary(s1a$risk.ill.1a)
tors1a <- tornado(s1a)
s1a.tor <- as.data.frame(print(tors1a)[[1]])

#S1B: vomit in bathroom, ESW spreads to ward----------------
summary(s1b$risk.ill.1b)
tors1b <- tornado(s1b)
s1b.tor <- as.data.frame(print(tors1b)[[1]])

#S2A: diarrhea in bathroom, ESW gets sick----------------
summary(s2a$risk.ill.2a)
s2a.1 <- as.data.frame(summary(s2a$risk.ill.2a)[[1]])
```

```
tors2a <- tornado(s2a)
s2a.tor <- as.data.frame(print(tors2a)[[1]])

#S2B: diarrhea in bathroom, ESW spreads to ward----------------
summary(s2b$risk.ill.2b)
tors2b <- tornado(s2b)
s2b.tor <- as.data.frame(print(tors2b)[[1]])

#S3A: vomit in room, ESW gets sick----------------
summary(s3a$risk.ill.3a)
tors3a <- tornado(s3a)
s3a.tor <- as.data.frame(print(tors3a)[[1]])

#S3B: vomit in room, ESW spreads to ward----------------
summary(s3b$risk.ill.3b)
tors3b <- tornado(s3b)
s3b.tor <- as.data.frame(print(tors3b)[[1]])

#S4A: diarrhea in room, ESW gets sick----------------
summary(s4a$risk.ill.4a)
tors4a <- tornado(s4a)
s4a.tor <- as.data.frame(print(tors4a)[[1]])

#S4B: diarrhea in room, ESW spreads to ward----------------
```

```
summary(s4b$risk.ill.4b),

tors4b <- tornado(s4b)

s4b.tor <- as.data.frame(print(tors4b)[[1]])
```

CHAPTER FIVE:

Conclusions, Public Health Significance, and Future Directions

Microbial infections place a significant health burden on society and are an important area of research. In particular, diarrheal illnesses kill thousands of people ever year, the majority of whom are children under the age of five (1,2). The leading cause of these diarrheal illnesses is human norovirus (HuNoV) which in one year will kill 200,000 people, infect 685 million, and cost the global economy $64.5 billion (3-5). This extremely infectious virus has no specific treatment or vaccine (5). In absence of medical interventions, engineering and behavior controls to prevent HuNoV are necessary. Many of these controls require measuring or modeling the presence of HuNoV in the environment as a means to target interventions, measure efficacy of disinfection, or predict health threats with risk assessments (6-8). However, since the first documentation of a HuNoV outbreak in 1968, the study and prevention of these viruses has been significantly impeded by an inability to grow HuNoV in any known laboratory cell line (9,10). With the lack of a cell culture model for infectivity, HuNoV detection has relied solely on detection of viral RNA using molecular tools, predominately by the use of reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (11). In particular, the use of genome equivalents (GE) as a surrogate for

viral copy numbers has been used to infer viral levels. Though these molecular methods are rapid, sensitive, and easily quantifiable, they remain unable to provide an accurate measure of infectious HuNoV as GE only represent the target nucleic acid segment that was amplified by RT-PCR (10). GE cannot be used to determine the infectious nature of isolated virus which limits the value when focusing on public health outcomes.

The development of the first reproducible method for culturing HuNoV *in vitro* was reported in 2016 which revolutionized the study of HuNoV (12). This novel cell culture method uses human intestinal enteroids (HIEs) which are 3D cultures that recapitulate the human intestinal epithelium (12,13). To date, researchers have applied this HIE culture approach to make strides in our knowledge of the physiology of HuNoV infections, in vaccine development, and in improved clinical detection (14-18,12,19,20). Another important area of HuNoV research that can benefit from the HIE method is environmental monitoring applications. This includes identifying the virus in food, water, air, and on fomites in order to predict human health risk and inform interventions. It is important to note that although the HIE approach can identify infectious viruses present in a sample, there is still the need to integrate RT-qPCR GE measurements for data output. This is done by comparing GE at one-hour post infection to 72-hours post infection via RT-qPCR. The current HIE system is not capable of producing conventional

infectivity outcomes which include plaque assays or most probable number (MPN) estimates using cytopathic effects.

To date, most applications of the HIE method for culturing HuNoV have been focused on clinical applications (14,17,19,20). Adaptation of the HIE method to environmental monitoring efforts for HuNoV poses a unique set of challenges compared to existing work using HIEs. The goal of this dissertation was to address some of these challenges and build a foundation for applying the HIE model in environmental monitoring scenarios. This was achieved through three specific research aims: (1) establish a set of research-based methodological recommendations for environmental sampling applications of the HIE system, (2) pilot the HIE system for surface recovery of HuNoV, and (3) develop a quantitative microbial risk assessment (QMRA) that can be a template for translating infectious HuNoV data from HIEs to public health interventions.

The first of these research goals was described in Chapter 2, “Optimizing human intestinal enteroids for environmental monitoring of human noroviruses.” In this chapter, I systematically examined six experimental variables of the HIE HuNoV assay: HIE age, HIE monolayer basement membrane, HuNoV inoculum processing, HuNoV inoculum volume, handling of data below limit of detection (LOD), and cutoff for positive samples. I found that HIEs were able to successfully replicate HuNoV with robust replication for all three age scenarios

that were examined: passage out to 58, 187 days of continuous growth, and after 334 days of enteroids archived in liquid nitrogen. Basement membrane and HuNoV processing methods were not associated with a change in HuNoV replication in HIEs. Higher inoculum volumes did not impact qualitative detection of infectious HuNoV and volumes close to the well maximum (250 microliters per well) are suggested to facilitate testing of larger volume samples in environmental applications. This finding will be incredibly valuable when examining low levels of virus present in many environmental locations. LOD replacement and fold cutoff values affected the qualitative measure of infectious HuNoV and should be explicitly stated in future work. This chapter also presented a list of data-informed best practices for experimental variables for the cultivation of HuNoV in HIEs, including a survey of current practices in the literature. The recommendations from this chapter can inform future applications of the HIE system for growing HuNoV, particularly those with the goal of environmental monitoring.

The second research aim was addressed in Chapter 3: “Recovery of infectious human noroviruses from fomites via replication in intestinal enteroids.” This work applied the HuNoV HIE cell culture method to measure infectious HuNoV recovered from fomites. Three fomites — a laminate hospital bed tray, a metal door handle, and a plastic sanitizer dispenser — were inoculated with HuNoV positive stool suspensions and swabbed to recover HuNoV. Successful

cultivation of fomite-recovered HuNoV in HIEs occurred from surfaces inoculated with at least 10^5 HuNoV GE/ cm^2. The highest recovery of infectious HuNoV was from surfaces inoculated with a high viral titer (>10^6 GE) in 50µL of inoculum. No difference was observed in recovery of infectious HuNoV across the three fomites. This is promising for future environmental monitoring work as it appears that multiple types of fomites can be swabbed for infectious HuNoV. Of note, I found that even in scenarios with low molecular recovery of HuNoV RNA (<0.1%), infectious HuNoV particles could still be collected from fomites and grown in HIEs. In a subset of swabbing experiments, I also tested recovery of the surrogate virus MS2 coliphage. MS2 have been used as HuNoV surrogates for decades and including this surrogate while developing a new approach is standard practice (21,22). Recovery of MS2 was comparable between molecular and infectivity methods, which provides validity to my experimental set-up. MS2 recovery (74%) was 2-fold greater than molecular HuNoV recovery (0.74%), which is consistent with previous evidence that MS2 inactivation may not be comparable to HuNoV inactivation (21-23,10,24-26). Literature has suggested that MS2 may be more resistant to inactivation than HuNoV which can be protective of human health but may overestimate inactivation needs, leading to waste of resources (23). HIEs will grow fomite-recovered HuNoV and inconsistency in results obtained with the surrogate virus MS2, compared to those obtained for HuNoV in HIEs, further justify the time and effort required to grow HuNoV in HIEs. Though the HIE method remains an imperfect tool, my

work offers an approach to move forward with fomite monitoring and disinfection studies.

The third research aim of this dissertation was addressed in Chapter 4: "Quantitative microbial risk assessment of human norovirus infection in environmental service workers due to healthcare-associated fomites." In this chapter I developed a quantitative microbial risk assessment (QMRA) looking at HuNoV infection risks from fomite contacts posed to environmental service workers (ESWs) in healthcare settings. For a single fomite contact in a room where a patient experienced a diarrhea event, the risk of HuNoV infection for ESWs was as high as 1:3. For scenarios where the ill patient vomited without a diarrheal event, the estimated risk to ESWs from one fomite contact dropped significantly – to 1:23,928 for an in-bathroom event and 1:51,504 for an in-room event. For secondary HuNoV infection from contact with a contaminated fomite, risks were 32% lower than ESW infection risks in diarrhea scenarios and 80% lower than ESW infection risks in vomit scenarios. The high risk estimates in diarrhea scenarios indicate that transmission of HuNoV from diarrhea may be primarily driven by fomite transfer, as compared to airborne or person-to-person transfer from vomit events. I demonstrated that a single fomite contact event can lead to high infection risk in ESWs and that ESWs may be able to spread HuNoV to other ward surfaces and initiate secondary infections. This study adds to the growing body of literature that points to fomite transmission as a significant

pathway in the spread of HuNoVs (27-31). ESWs likely face important occupational health risks from fomite-mediated infections and interventions are needed to both protect them and prevent further spread.

Taken together, the research presented in this dissertation provides a strategy for using the HIE system to cultivate HuNoV recovered from the environment and presents a QMRA model that can be integrated with future measures of infectious HuNoV. The main limitations of this work are ongoing challenges with the HIE method for HuNoV cultivation and low data availability for constructing QMRA models. For chapters 2 and 3, the inability to directly quantify HuNoV grown in HIEs makes comparisons challenging and prevents the calculation of percent recovery. In the future, methods to quantify HuNoV growth in HIEs should be explored, such as the MPN method. For Chapter 4, much of the data required for the QMRA was not explicitly available and multiple assumptions had to be made. Additionally, the data used for the QMRA was not norovirus specific in many cases and instead was pulled from literature on other pathogens or surrogate viruses.

This work contributes a number of novel findings to the body of HuNoV literature. In Chapter 2, I investigated a series of methodological variables in the HIE system and their impact on HuNoV growth. This work is the first to examine HIE cell age, basement membrane, viral inoculum processing, viral inoculum volume,

LOD handling, and choice of fold cutoff and offers a set of evidence-based recommendations that can improve the use of the HIE system to grow HuNoV. In Chapter 3, I demonstrated that HuNoV can be recovered from fomites and successfully grown in HIEs. This is the first work to use the HIE system with fomite recovered viruses and serves as a foundation for future work on growing HuNoV recovered from the environment. Finally, Chapter 4 presented a QMRA of HuNoV infection risk from fomites posed to ESWs. This QMRA is the first to examine these specific scenarios and serves to enhance the literature on HuNoV and healthcare QMRAs. The model I produced in Chapter 4 is a template for future QMRAs and provides a framework to integrate HuNoV infectivity data from HIEs in the future.

HuNoV poses a significant health burden and leads to a large number of illnesses, loss of life, and substantial economic losses. This dissertation addressed one of the main challenges in HuNoV research – the inability to culture HuNoV in vitro. The development of the technique to grow HuNoV in HIEs opened the door for new research that could better relate HuNoV detection to infectious viruses and, ultimately, health outcomes. This body of work demonstrates how the HIE cell culture can be used in environmental detection of HuNoV moving forward. Through the development of methods, application of new techniques, and inclusion of a QMRA, I was able to illustrate how to use the

HIE method to translate measurement of infectious HuNoV from the lab bench to human health outcomes.

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